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The role of sex on behavioral responses to mating signals: Studies of phonotaxis and evoked calling in male and female túngara frogs

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The role of sex on behavioral responses to mating signals: Studies of phonotaxis and evoked calling in male and female túngara frogs

by

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Dedication

To A. Stanley Rand,

for sharing with me his endless passion for understanding nature, and

teaching me the great value of a detailed observation of the natural world

To Andres,

for his unconditional love and support.

Thank you for always having a smile for me!

To Tomas,

whose arrival inspired me during my last year in graduate school

To my parents,

who taught me to pursue my dreams

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Ximena E. Bernal

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The role of sex on behavioral responses to mating signals: Studies of phonotaxis and evoked calling in male and female túngara frogs

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Signal detection theory predicts that costs associated with recognition errors, specifically failing to respond to relevant stimuli (missed detection) and responding to erroneous ones (false alarms), shape receiver permissiveness in animal communication systems. Fitness costs of missed detection and false alarms in response to sexual signals differ between the sexes, and are usually higher for females than males. This asymmetry in costs predicts that males should be more permissive than females in their responses to signals. In my dissertation I investigate the behavioral responses of male and female túngara frogs, *Physalaemus pustulosus*, to mating signals and sounds associated with such calls. Specifically I explore the following topics: i) responses of the sexes to call complexity, ii) perception of congeneric mating calls by males and females, iii) responses of males to the conspecific call compared to those of extant heterospecifics, iv) effect of

sounds associated with increased predation risk in reproductive decisions, and v) effect of the task performed by each sex on signal permissiveness. My findings indicate that recognition errors are higher for males than females as predicted by the different costs associated with recognition errors for each sex. Males respond to a broader range of calls than females. Despite the differences, evolutionary history has left a footprint on the brain of both sexes. In addition, I found that females behaved more cautiously than males suggesting that the sexes balance the risk of predation and the cost of cautious mating strategies differently. In the mating system of túngara frog, as in many others, sexual signals elicit different tasks in the different sexes, female phonotaxis and male calling. Therefore, the sexual differences in decision making I found could be either sex-specific independent of task, or task-specific independent of sex. Here I show that sexual differences in receiver permissiveness are motivated by differences due to the typical reproductive tasks displayed by the sexes.

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Introduction

Signal detection theory predicts that costs associated with recognition errors, specifically failing to respond to relevant stimuli (missed detection) and responding to erroneous ones (false alarms), shape receiver permissiveness in animal communication systems. Fitness costs of missed detection and false alarms in response to sexual signals differ between the sexes, and are usually higher for females than males. A female basing a mate choice on the call of the wrong species, for example, could forfeit its reproductive investment for a substantial part of the breeding season, while a calling male that is engaged in such error would bear less dire consequences, perhaps merely wasted time and effort. The asymmetry in costs of errors in response to mating signals predicts that males should be more permissive than females in their responses.

In many species males advertise to both male and female audiences (McGregor & Dabelsteen 1996; Searcy & Nowicki 2000; Gerhardt & Huber 2002). For example, male crickets, frogs, and birds often use the same acoustic signal to attract females and to repel males. As mentioned above, males and females will not necessarily have the same threshold for responding to the same signal, especially when these signals are associated with reproduction (Trivers 1972). Few studies, however, have evaluated permissiveness of males and females to sexual signals. My dissertation investigates the behavioral responses of male and female túngara frogs, *Physalaemus pustulosus*, to mating signals and sounds associated with such calls.

The first chapter of my dissertation analyzes the responses of the sexes to call complexity. In túngara frogs, males produce a frequency modulated call (whine) to which they facultatively add from zero to up to seven broad frequency secondary components (chucks). I explore the natural dynamics in this call repertoire, and examined the phonotaxis responses of females and the evoked vocal responses of males to mating calls with varying number of chucks. Female túngara frogs are preferentially attracted to calls with one chuck than to simple whines, and males also call more in response to calls with one chuck. There is not evidence, however, for the function of increasing the number of chucks within complex calls. I found that males gradually change call complexity by the addition or subtraction of a single chuck without generally incurring in jumps to achieve greater complexity. In the wild, males rarely produce more than two chucks. The behavioral responses of males and females to calls with different number of chucks suggest that one reason for not escalating chuck number more drastically is that there is a diminishing return in the number of chucks on the response of both sexes.

In the second chapter of my dissertation, I explore the perception of congeneric mating calls. I examine the evoked vocal responses of male túngara frogs to male mating calls of conspecifics and fourteen species of heterospecific and ancestral calls to evaluate the influence of call similarity and phylogenetic distance on their responses. I also compare the vocal responses of male to female phonotactic responses to examine the propensity of response errors between the sexes. Recognition errors were higher for males than females as predicted by the different costs associated with recognition errors for each sex. Males responded to the calls of most species with mating calls, and

produced aggressive calls in response to two other heterospecific/ancestral calls. The responses of males were explained by phylogenetic distance but not by overall call similarity. Similarly, females are more likely to exhibit phonotaxis to calls of species and ancestors that are more closely related. Therefore, evolutionary history has left a footprint on the brain of both sexes but the details seem to differ.

The third chapter extends the previous one contrasting the evoked vocal responses of male túngara frogs to the advertisement call of their own species with those in response to seven extant heterospecifics. In chapter two I showed that male túngara frogs recognize the mating calls of a broad range of congeneric species, but it was unclear how the vocal response to heterospecific signals compares to the response to the conspecific call. In this chapter I test the hypothesis that the conspecific call elicits the greatest vocal response, as expected if the signal has evolved to elicit maximum stimulation in the receiver. I found that the greatest response was not elicited by the conspecific call but by the call of sp. B, a species in the *P. pustulosus* species group. Greater evoked vocal response to a heterospecific call than to the conspecific signal probably represents a response to supernormal stimulus. I also contrast the response function of males to the calls of their congenerics to previously published response functions of female túngara frogs to the same set of signals. Males' response function has a wider breadth and different shape than the one of females. This sexual difference in response functions represents different process of stimulus generalization probably due to different selective pressures on males and females.

In the fourth chapter of my dissertation I investigate the effect of sounds associated with increased predation risk in reproductive decisions of male and female túngara frogs. Here I investigate whether the acoustic cues associated with predatory attacks or acoustic cues associated with predators themselves affect the mating behavior of female and male túngara frogs. I compared the responses of females approaching a mate and those of calling males when exposed to mating calls associated with sounds representing increased hazard. When presented with mating calls that differed only in whether or not they were followed by a predation-related sound, females preferentially approached the call alone. In contrast, calling males showed greater vocal response to calls associated with increased risk than to a call by itself. I found significant differences in the responses of females and males to several sounds associated with increased hazard. Females behaved more cautiously than males suggesting that the sexes balance the risk of predation and the cost of cautious mating strategies differently.

Finally, the fifth chapter of my dissertation focuses on disentangling the effect of sex from the task performed by each sex to explore whether there are fundamental differences between males and females. In the mating system of túngara frog, as in many others, sexual signals elicit different tasks in the different sexes, female phonotaxis and male calling. Sexual differences in decision making could be either sex-specific independent of task, or task-specific independent of sex. The results show that males calling back in response to advertisement calls are less discriminatory than females approaching such calls. Males, however, moving towards the same stimuli behave as selective as females. Thus sexual differences consistent with a paradigm of highly

selective females and less discriminatory males are motivated by differences due to the typical reproductive tasks displayed by the sexes.

Chapter 1: Mating calls in túngara frogs, *Physalaemus pustulosus*: the paradox of greater call complexity

Abstract. Mating displays involving multiple components are often interpreted to have evolved by sexual selection. Large call repertoires have received ample attention in songbirds, while few studies have examined this phenomenon in anurans. We investigate the natural dynamics in a repertoire that combines different call notes, and then explore the role of these phenotypes in male-female and male-male interactions in a Neotropical frog. Male túngara frogs, *Physalaemus pustulosus*, produce frequency modulated mating calls to which they can facultatively add up to seven short, multi-harmonic components known as chucks. Female túngara frogs are preferentially attracted to calls with chucks over simple whines, and males also call more in response to calls with chucks. Frog-eating bats and blood-sucking flies are also preferentially attracted to calls with chucks over calls simple whines. In the context of simple (no chucks) versus complex (any number of chucks) calls, the variably complex call appears to have evolved in response to the counter-selection forces of natural and sexual selection. There is not evidence, however, for the function of increasing the number of chucks within complex calls. We tested two aspects of increasing call complexity: how males use calls with multiple chucks in the wild, and how males and females respond to calls with multiple chucks. We found that males incrementally change call complexity by the addition or subtraction of a single chuck. In the wild, males rarely produce more than two chucks. We also found that males and females responding to male calling do not distinguish between calls with one

or more chucks. Our results suggest that one reason for not escalating chuck number more drastically is the diminishing return in the number of chucks on the response of both males and females.

1.1. Introduction

In many species males use multiple ornaments and displays to attract females. Multi-component sexual displays within a single sensory modality include complex acoustic mating systems such as the song and note repertoires in songbirds (Andersson 1994). For songbirds it has been proposed that large vocal repertoires are a product of sexual selection (Catchpole 1980; Krebs and Kroodsma 1980). Female great reed warblers, for instance, preferentially solicit copulations from males with large syllable repertories (Catchpole et al. 1986), and females paired with males with small repertoires engage in extrapair copulations with neighbor males displaying larger repertories (Hasselquist et al. 1996). Alternatively, large song repertoires can also confer an advantage in intrasexual competition to gain and hold territories. In song sparrows (Hiebert et al. 1989) and great tits (Krebs et al. 1978) large repertoires correlate with increased ability to proclaim a territory. Hiebert et al. (1989) also show that holding territories longer translates into greater annual and lifetime reproductive success. Thus, a complete understanding of how sexual selection shapes repertoire size requires integrating the selective pressures imposed by both intended receivers of these signals, males and females. Here we investigate the natural dynamics in a repertoire that combines different call notes, and

then explore the role of these phenotypes in male-female and male-male interactions in a Neotropical frog.

In most anurans, males produce a stereotyped mating call that is repeated with little variation for several hours at their breeding areas (Gerhardt and Huber 2002). These redundant calls potentially reduce recognition errors and increase localizability. In some frogs, however, males produce mating calls with large repertoires (Schwartz and Wells 1984b; Schwartz and Wells 1984a; Ryan 1985; Schwartz and Wells 1985; Narins et al. 2000). The túngara frog, *Physalaemus pustulosus*, has a call that varies in complexity in a way unusual for frogs and toads (Rand et al. 1981). Males produce a frequency modulated whine to which they can facultatively add secondary multi-harmonic ornaments called chucks (Fig. 1; Rand and Ryan 1981; Ryan 1985). Chucks are produced by a fibrous mass attached to the vocal folds which probably evolved by sexual selection (Gridi-Papp et al. 2006). While whines can be produced alone, chucks are always attached to whines. Males produce whines without chucks most often when they are calling by themselves, while calls with chucks are more common in reproductive assemblages with several males. Males can add from zero to seven chucks to a whine (Bernal et al. 2007). In túngara frogs we refer to a repertoire as the variable number of elements in the mating song rather than to the number of mating songs, as is also commonly used in birdsongs.

We know female túngara frogs are preferentially attracted to calls with chucks over simple whines, and males also call more in response to calls with one chucks (Rand and Ryan 1981; Ryan and Rand 1998). Frog-eating bats (Ryan et al. 1982) and blood-

sucking flies (Bernal et al. 2006) are also preferentially attracted to calls with chucks over simple whines. Thus the variably complex call appears to have evolved in response to the counter-selection forces of natural and sexual selection, at least in the context of simple (no chucks) versus complex (any number of chucks) calls. There is no evidence, however, for the function of increasing the number of chucks within complex calls; that is, why males ever produce more than one chuck.

In the wild about half of the calls produced by male túngara frogs have chucks. The majority of those complex calls have one chuck, calls with two chucks are less common while calls with three or more chucks are rare (Bernal et al. 2007). Despite our recent increase in knowledge about the natural signal variation at choruses in the wild, it is unclear how males escalate the complexity of their calls, and whether males enjoy any advantage when producing more than one chuck. In this study we quantify how males use calls with multiple chucks in their call bouts, and test the hypothesis that greater call complexity increases call response in rival males and/or call attractiveness to females.

1.2. Methods

Male and female frogs were collected during the rainy season, between May and August 2000-2006, at breeding ponds around the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07.0'N, 79°41.9'W). All frogs were brought to the laboratory, and subsequently returned to their capture sites. Prior to returning the frogs, we gave them a unique toe-clip number to prevent them from being tested or their calls recorded again.

Sequence analysis of call complexity

Túngara frogs call in bouts in which a few males initiate calling, and then other males join them until the chorus reaches a peak of calling activity, after which individual frogs cease calling until all are silent. The entire process starts anew when a few leading males begin to call (Pauly et al. 2006). We recorded 90 call bouts of individual focal males using a WM-D6C Sony Tape recorder and Sennheiser ME-66 shotgun microphone. The microphone was placed 1 m from the calling frog, and all males were captured, measured for snout-vent length and marked after recording their calls.

For each male we categorized their calls according to the number of chucks added to each whine and the sequence in which these variably complex calls were produced. We then analyzed this vocal sequence to understand how males transition between calls of varying complexity. We organize preceding and following call types into a transition probability matrix in which each cell represents the total intances of call type j following call type i (Table 1). We calculated the expected value for each transition by multiplying the column frequency by its corresponding row total. To establish if there is an association between preceding and following call types we used a chi-square goodness of fit using the Yate's correction for continuity (Zar 1996). The total chi-square value for the entire matrix is equal to the sum of the total row chi-square values. From the transition matrix it was necessary to establish which of the dyads in a row were significant, to do so we used a modified chi-square value with 1 degree of freedom per cell (Clark 1994). When calculating the transition probabilities between call types we did

not include cells representing call transitions that did not incur changes of chuck number because we are interested in the transitions that generate changes in call complexity. Transition probabilities were calculated by dividing the total instances of a given call type by the corresponding row total.

Behavioral response of males and females to increased complexity

Acoustic stimuli

As experimental stimuli we used whines with a variable number of chucks, ranging from zero to six. Using individuals of both sexes we performed one set of experiments with synthetic calls, and a second one with natural calls. Evaluating the behavior of túngara frogs to natural calls allowed us to explore any effects undetected in the experiments using synthetic calls. Synthetic versions of the average whine for the population successfully elicit phonotaxis in females (Ryan and Rand 1990) and calling behavior in males (Bernal et al. 2007). Synthetic calls offer high internal validity but do not completely replicate natural calls. Synthetic and natural túngara frog calls differ in several traits (Fig. 1.1). Natural whines, for instance, have a series of harmonic frequencies instead of having only the fundamental frequency (also the dominant frequency). Previous studies have shown that females do not discriminate on average between natural and synthetic whines.

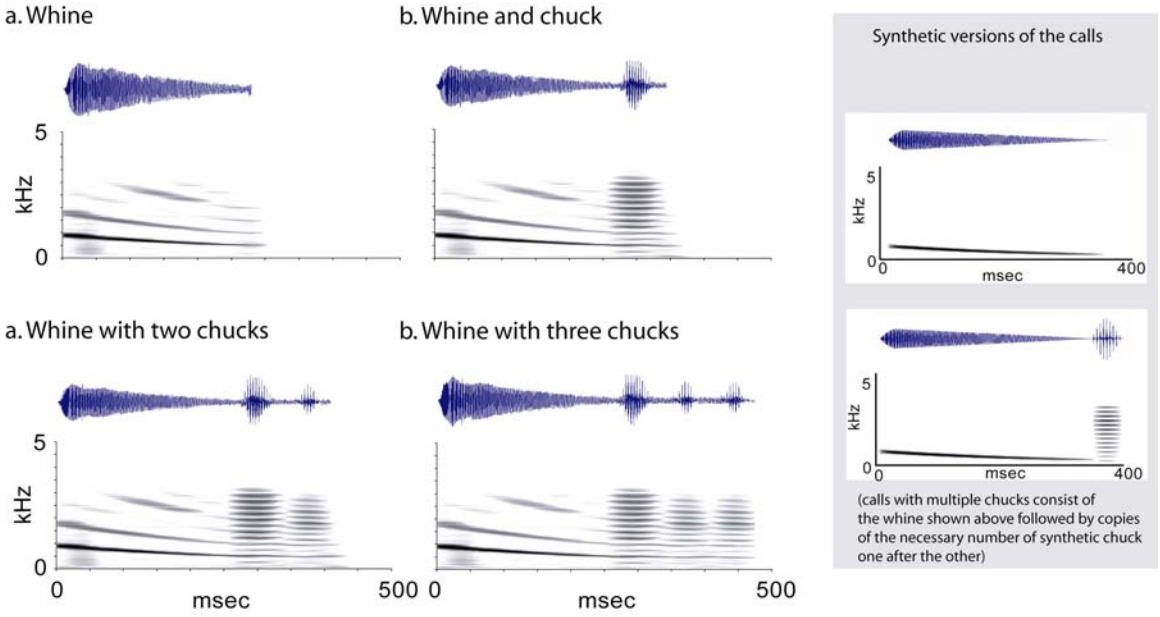

Figure 1.1. Complexity series of the advertisement call of *Physalaemus pustulosus*, the túngara frog. (a-d) Natural versions of the calls are shown with zero to three chucks. Synthetic versions of the calls with zero and one chuck are presented in the box on the side for comparison.

We synthesized the calls based on the mean values of the parameters of the calls in the population by shaping sine waves using the software developed by J. Schwartz (Pace University at Pleasantville, NY; sample rate 20 kHz and 8 bit). Mean values for the population were calculated based on the calls from 50 males recorded in July 1996 with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003).

The natural calls are recordings of calling males from the field or the laboratory. The calls used in the experiments with the males were recorded in the laboratory in individual acoustically-isolated chambers with a Sony WM-D6 cassette recorder and a Radio Shack miniature condenser microphone. The chambers were lined with sound

absorbent material to minimize echo and attenuate sounds arriving from outside the chambers. We chose recordings with minimal background noise from 10 males that produced calls with different numbers of chucks. In most cases males added from zero up to two chucks to a whine, but a few males produced three chucks. To obtain whines with three and six chucks for each male we digitally duplicated the terminal chuck of the call and appended it at the end as many times as necessary to achieve the desired number of chucks for the experiment.

The natural calls used in the experiments with the females were recorded in the field with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. We selected the calls of 20 males based on previous studies of female preference using the same calls (details in Ryan and Rand 2003). The call of each male was modified to obtain the desired number of chucks for each test duplicating the initial chuck of the call and appending it to the end of the whine as many times as required. We used each pair of calls of different call complexity from each male only once.

Male Evoked Vocal Response Experiments

In 2003 and 2004 calling males were captured at their breeding sites and brought to the lab where each male was placed inside individual acoustically-isolated chambers (30.5 cm x 46 cm x 30.5 cm) following Bosch et al. (2000b; 2002) and Bernal et al. (2007). To record their calling behavior we placed males with sufficient water to call in plastic bags previously shown to be acoustically transparent by Ryan & Rand (1998). We broadcast

the experimental stimuli using a small, wide-frequency range speaker (Cambridge SoundWorks Inc., Ensemble IV) and recorded the response of the males with a Radio Shack miniature condenser microphone into a Sony WM-D6 cassette recorder. We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 μPa) at 0.5 m measured by a GenRad sound pressure level meter model 1982. We digitized the tapes using CoolEdit 2000 (Syntrillum Software Corporation), at a sampling rate of 44.1 kHz and 16 bits/sample.

We tested males in evoked vocal response experiments using both synthetic and natural calls. In 2003 we used synthetic versions of the average whine and chuck in the population (see section on acoustic stimuli). In 2004, we performed a second series of tests using natural calls to further explore the responses of males to calls with multiple chucks. In this second experiment, in addition to using natural calls we used a more robust experimental design that would allow us to account for variability between males to exhaust the possibilities of finding an effect of call complexity. We recorded whines with variable numbers of chucks from ten males and presented each male's complexity series in random order to a single male (see section on acoustic stimuli). We tested a total of ten males, each one with the calls of a randomly selected but different male, for a repeated measures design.

In both kinds of evoked vocal response experiments we used the same methodology to present the stimuli. We stimulated the males with a túngara frog chorus recorded on 2 October 1990 from the same population. Once a male was calling, we tested him singly. Each test consist of a set of five 60-second intervals in the following order: i) Control

stimulus: white noise shaped with the amplitude envelope of the whine (0-10 kHz), ii) Silence, iii) Experimental stimulus: whine with 0, 1, 2, 3 or 6 chucks, iv) Silence, and v) Control stimulus (see also Ryan and Rand 1998; Bosch et al. 2000a; Bernal et al. 2007). After a male finished a test, it was required to call again before starting the next test. Males were tested until they participated in all the experimental stimuli or ceased calling. We broadcast the calls at a rate of one call every two seconds, a typical calling rate for túngara frogs (Ryan 1985). Additional details are presented in Bernal et al. (2007).

To evaluate male calling behavior we counted the number of whines, number of chucks and maximum number of chucks in a single call produced by each male in response to the experimental and control stimuli. To account for any changes in male motivation during the experiments we examined the strength of male calling in response to each stimulus by averaging the calling response of the two controls to calculate the ratio of responses: experimental/(average control +1) following Bosch et al. (2000a, 2002). We then characterized the overall response of males using the first component of a principal component analysis (PCA) combining the ratios of responses in number of whines, number of chucks, and maximum number of chucks in a single call. We performed separate PCA for the experiment using synthetic calls and the one using natural calls, thus independent measurements of overall calling response were obtained for each set of tests. In the experiment using synthetic calls (between groups design), we evaluated the effect of adding chucks to a whine on male calling behavior with a Kruskal–Wallis ANOVA followed by post hoc comparisons with Dunn's test using SigmaStat (Systat Software Inc, California, USA). For the data obtained using natural

calls (repeated measures design) the Friedman test was used and Dunn's test For was used for post hoc comparisons.

Female Phonotaxis Experiments

In 2000 and 2006 we performed standard two-speaker phonotaxis tests offering females a choice between calls that differed in the number of chucks appended to a whine. We placed each female under a funnel in the center of a 1.8 m x 2.7 m sound attenuation chamber (Acoustic Systems, Austin, TX), while the stimuli were broadcast from speakers placed in the center of walls opposite one another. After 3 min, we remotely removed the funnel allowing the female to freely move in the arena. We broadcast the test stimuli antiphonally such that the peak amplitude of the whine of each test call at the center of the arena was 82 dB SPL (re. 20 mPa). Speakers were balanced for sound pressure level using a 500 Hz continuous tone. We scored a choice when the female approached within 10 cm of either speaker. If a female was motionless at the release point for 5 min, did not move for any 2 min span of time after exiting the releasing point, or spent more than 15 min roaming the arena without approaching a speaker, no choice was recorded. We observed the behavior of the females on a monitor using a wide angle lens video system with an infrared light source (Fuhrman Diversified, Inc).

We tested females in phonotaxis using both synthetic and natural calls. In 2000 we used synthetic calls created based on the mean values of the call parameters for the population. We evaluated the responses of females to all possible combinations of calls with zero to three chucks, for a total of six paired tests (0 vs. 1, 0 vs. 2, 0 vs. 3, 1 vs. 2, 1

vs. 3, 2 vs. 3). In 2006 we further explored the potential attractiveness of multiple chucks by examining female choice in response to natural calls. Details about the natural and synthetic calls can be found in the *acoustic stimuli* section.

We used an exact binomial test for each pair of stimuli to determine the effect of call complexity on female mate choice. Because we predicted that calls with more chucks are preferred when contrasted with those containing fewer chucks we used a one-tailed probability. We also combined the outcome of the experiments using synthetic and natural calls to obtain an overall significance test for the effect of call complexity using the Fisher's method for combining probabilities (Sokal and Rohlf 1995).

1.3. Results

Sequence analysis

On average males produced call bouts consisting of 32.63 calls (SEM= 3.58) with a broad range of variation (min value = 4 calls, max value = 255 calls). The maximum number of chucks appended to a whine was three. Approximate one quarter of males did not produce complex calls (25.6 %), and these males had shorter bouts than males that increased call complexity (t = -3.349, df = 88, P = 0.001).

The degree of call complexity was dependent on the complexity of the preceding call (x^2 = 4196.39, df = 9, P < 0.001, n = 90; Table 1.1). This was true even when cells including transitions to the same state were excluded from the analysis (x^2 = 381.30, df = 9, P < 0.001, n = 90). Estimating the chi-square value for each cell of the matrix we extracted significant dyads, shown in bold in Table 1. For all call types, whines with the

same number of chucks (i.e. no change in complexity) were produced in greater frequency than expected. Transitions that produced increases and decreases of chuck number between zero and two chucks, however, were produced in lower frequencies than expected.

Table 1.1. Túngara frog call complexity transition matrix for whines with up to three chucks (n = 90 males). Top number of the row is the observed value (obs) and the bottom number is the expected value (exp). Row chi-square values using the Yates' correction are given in the far right column, and the chi-square value for the entire matrix is shown at the bottom of such column. Statistical significant dyads are shown in bold. ** $P < 0.001$

		Following call type (number of chucks)					
Preceding call type (number of chucks)		0	1	2	3	Row total	Row chi square
0	obs	**919****	**144****	**9**	0	1072	1118.66
	exp	**393.33**	**440.95**	**231.72****	6.00		
1	obs	**121****	**956****	**83**	0	1160	813.94
	exp	**452.62**	**477.15**	**250.75****	6.49		
2	obs	**9****	**76****	**519****	7	611	1460.59
	exp	**224.18**	**251.32**	**132.07**	3.42		
3	obs	0	0	7	**9****	16	803.18
	exp	5.87	6.58	3.46	**0.09**		
Total		1049	1176	618	16	2859	**4196.39****
Frequency		0.367	0.411	0.216	0.006		

The transition probabilities between calls with different number of chucks are shown in Fig.1.2. Transitions with high probabilities involve the addition or subtraction of a single chuck. Males producing single whines usually escalate by adding only one chuck. Calls with one chuck have a slightly higher chance of going back to zero chucks than appending an additional chuck. Males producing whines with two chucks are highly likely to reduce call complexity to one chuck in the next call. Whines with three chucks

are reached only from calls with two chucks, and also deescalate into whines with two chucks.

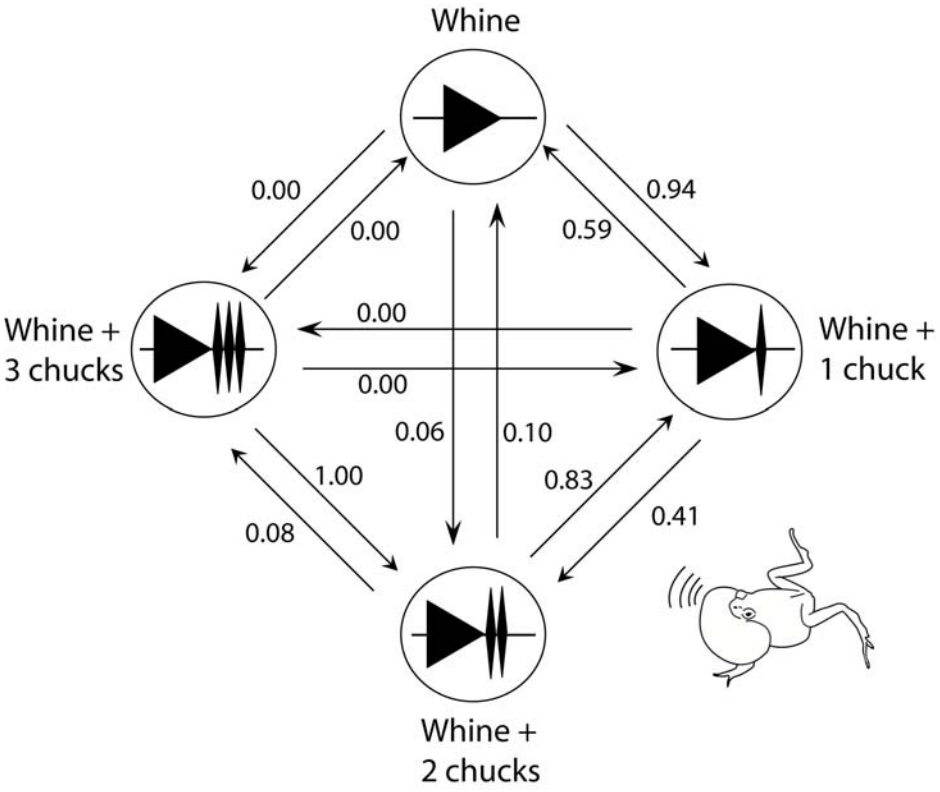

Figure 1.2. Sequence diagram of male túngara frog calling behavior. Numbers indicate the transition probabilities from one call type to the subsequent call type.

Male Evoked Vocal Response

Males exhibit a change in their calling behavior in response to call complexity (Fig. 1.3; synthetic calls: Kruskal-Wallis $H = 20.59$, $df = 4$, $P < 0.001$; natural calls: Friedman $Q = 23.12$, $df = 4$, $P < 0.001$). Their responses are similar in the experiments using synthetic and natural calls. In both cases, males call significantly more in response to a whine with any number of chucks than to a single whine ($P < 0.05$). Calling behavior, however, is not significantly different in response to calls with one, two or three chucks ($P > 0.05$). It

is possible that males reach the maximum calling response achievable given the experimental design (e.g., two seconds of silence between playback calls). Calls with six chucks, however, do significantly increase calling response over calls with up to three chucks using synthetic calls ($P < 0.05$), and up to two chucks using natural calls ($P < 0.05$). This suggests that the lack of increased response to two and three chucks is not due to a constraint in our design but to lower motivation.

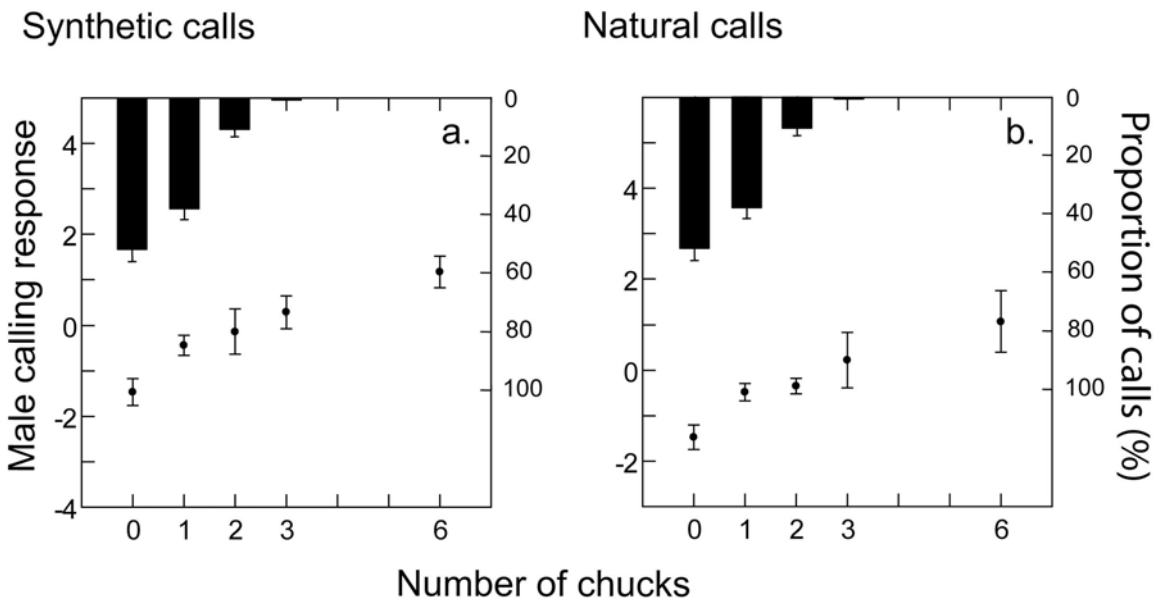

Figure 1.3. Male evoked vocal response to synthetic (a) and natural (b) calls that vary in number of chucks appended to the call. The letters indicate significantly different groups based on a Kruskal–Wallis ANOVA and post hoc Dunn's test. The bars on top and left y-axes represent the natural distribution of each call type in the wild adapted from Bernal et al. (2007).

Female Phonotaxis Response

Females strongly preferred a whine followed by chucks to a whine without any secondary components appended (Fig.1.4a, c). When using both natural and synthetic calls there was a trend for females to prefer calls with more chucks over those with fewer chucks

(Fig. 1.4b, d). The number of females, however, that preferred calls with more chucks beyond one chuck was not statistically significant for any of the tests (Table 1.2, Fig. 1.4). Power analyses show that, based on the responses of females in these experiments, we would need a sample size between 66-618 females to uncover significant differences (statistical power of 0.80).

On the basis of all the phonotaxis tests together, we can conclude that increasing the complexity increases attractiveness to females ($x^2 = 91.21$, $df = 22$, $P < 0.001$). This general trend, however, is generated by the strong preference of females for calls with any chucks over simple whines. In five of the six tests in which calls with more than one chuck were paired (1 vs. 2, 2 vs. 3, etc), more females were attracted to the whine with more chucks. But when considering only these tests together there is not a significant effect of a greater number of chucks beyond one ($x^2 = 14.99$, $df = 12$, $P > 0.05$).

1.4. Discussion

Male túngara frogs produce calls of varying complexity in response to the counter-selection forces generated by sexual and natural selection. Calls with chucks are more attractive to females but increase predation risk from frog-eating bats (Ryan et al. 1982) and parasitism risk from blood-sucking flies (Bernal et al. 2006). The reason males

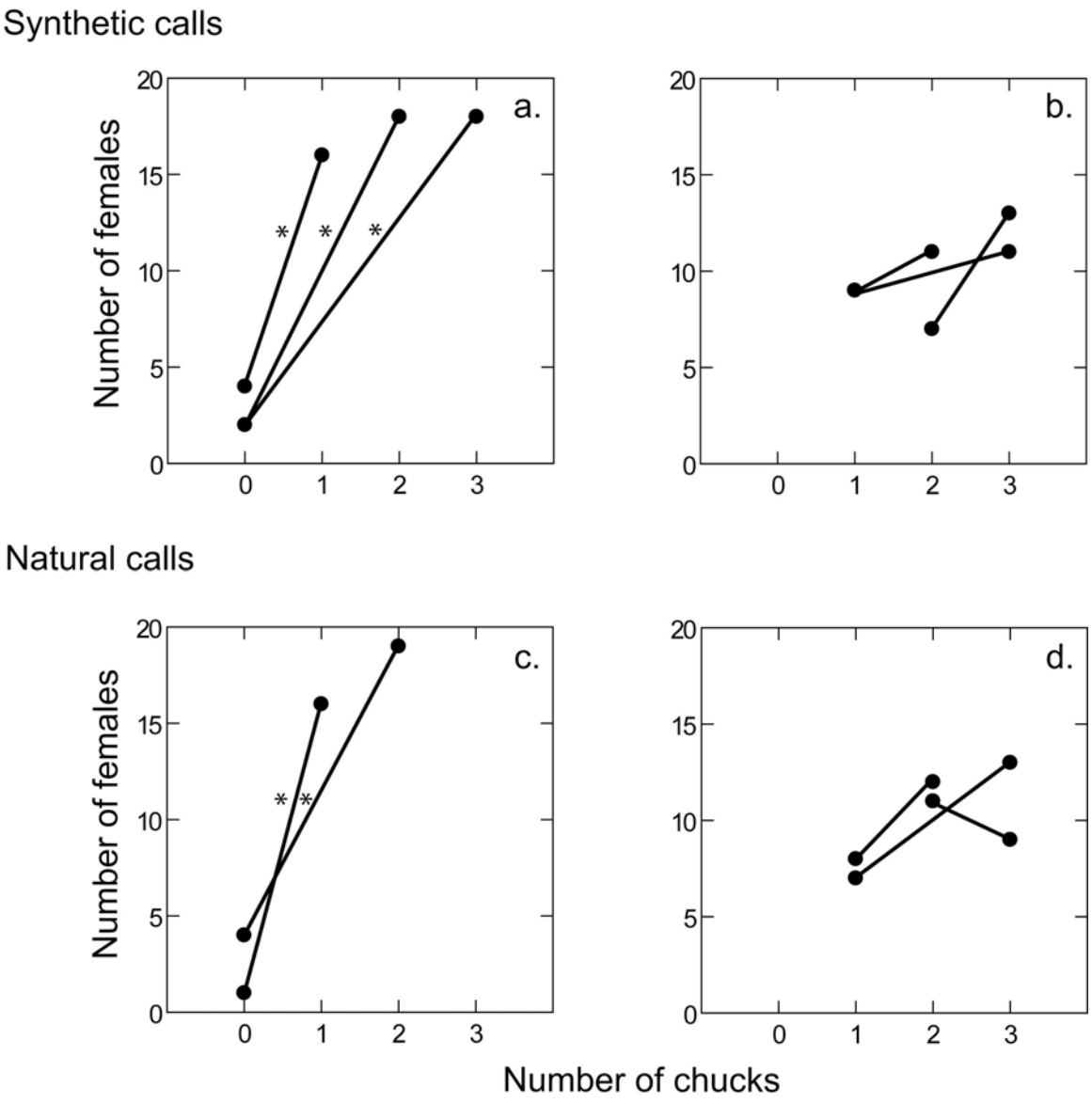

Figure 1.4. Female phonotaxis responses to synthetic (a, b) and natural (c, d) calls that vary in number of chucks appended to the call. Calls paired in each phonotaxis experiment are linked by a line. *P < 0.05 using a one-tailed exact binomial probability test that each pair of stimuli.

Table 1.2. Responses of túngara frog females in phonotaxis tests to calls that vary in the number of chucks appended. The choices represent the number of females attracted to less/more chucks (CH) appended to a whine (WH). *P* is the probability of rejecting the null hypothesis using a one-tailed exact binomial test; significant probabilities are shown in bold. For non-significant tests, the power of the test and the sample size required given the observed effect to achieve statistical power of 0.80 are also shown.

Test	Choices	*P*	Power	*n* (Power = 0.80)
Synthetic calls:				
WH 0CH vs. WH 1CH	4/16	**0.006**	-	-
WH 0CH vs. WH 2CH	2/18	**<0.001**	-	-
WH 0CH vs. WH 2CH	2/18	**<0.001**	-	-
WH 1CH vs. WH 2CH	9/11	0.412	0.055	618
WH 1CH vs. WH 3CH	9/11	0.412	0.055	618
WH 2CH vs. WH 3CH	7/13	0.132	0.375	66
Natural calls:				
WH 0CH vs. WH 1CH	1/19	**<0.001**	-	-
WH 0CH vs. WH 2CH	4/16	**0.006**	-	-
WH 1CH vs. WH 2CH	8/12	0.253	0.227	149
WH 1CH vs. WH 3CH	7/13	0.132	0.375	66
WH 2CH vs. WH 3CH	11/9	0.748	0.055	618

produce calls with more than one chuck, however, is not understood. Here we show that males gradually change call complexity by the addition or subtraction of a single chuck. Bernal et al. (2007) showed that ca. 78% of calls had zero or one chuck, while more than 99% of the calls had two or fewer chucks. Thus, although males can add up to seven chucks it is rare to produce more than two. The lack of calls with many chucks might be due to constraints in signal production. Our results, however, suggest that one reason for not escalating chuck number more drastically in the wild might be the diminishing return on the response of both males and females.

Dynamics of call complexity

Analysis of sequential calls reveals a gradual increase in call complexity in túngara frogs. Consecutive calls are highly likely to remain at the same degree of complexity and if not it most often will be due to the addition or subtraction of single chucks. Why is there a gradual change in call complexity? Chucks are produced by a fibrous mass that extends into the bronchus and is supported by the vocal folds (Gridi-Papp et al. 2006). There are no reasons to assume mechanical constraints that would force gradual escalation in call complexity, and our data shows that males are physically able to add or remove two chucks in a single transition, though they do so rarely. The pattern of escalation we find is probably shaped by the social environment. The call bouts recorded in the wild reflect male calling strategies when interacting in breeding aggregations. At choruses, males organize their calls into bouts in which one or two males initiate calling, and other males join them until the chorus reaches a peak of calling activity. This gradual addition of males to the chorus generates a slow increase in acoustic interactions leading to smooth escalations in call complexity in single males.

Increasing call complexity from zero to one chuck is highly probable while escalating beyond this point is much less likely. For whines with two or three chucks, de-escalation in call complexity is the trend. This is consistent with the fact that most calls produced in the wild have zero or one chuck (Bernal et al. 2007). These results stimulate questions about the payoffs of calls with more than one chuck. In the next section we address female attractiveness and male vocal responses to such calls, important components shaping call complexity.

Behavioral responses to call complexity

Our study confirmed that both males and females are more responsive to complex calls with one chuck than to a single whine. Moreover, we found that any number of chucks increases call attractiveness to females and calling response of males compared to a whine alone. Nonetheless, approximately half of the calls produced by túngara frog males in nature do not have chucks, however (Bernal et al. 2007). A relatively high production of simple calls is explained by the costs imposed by adding chucks to a whine (Ryan 1985). Specifically, despite the lack of an additional energetic cost of chucks production (Bucher et al. 1982), complex calls increase the likelihood of attacks by frog-eating bats (Ryan et al. 1982) and blood-sucking flies (Bernal et al. 2006).

When both sexes were exposed to calls with multiple chucks, neither females nor males were more responsive to calls with greater numbers of chucks. This lack of enhanced behavior is for both sexes unexpected. It is unlikely that there is a preference or increased vocal response that we did not detect. We used methodologies and sample sizes proved to be appropriate to evaluate female and male behavior in túngara frogs and other anurans (reviewed in Gerhardt and Huber 2002). Moreover, even though synthetic calls are successful at eliciting female phonotaxis (Ryan and Rand 1990; Ryan and Rand 1995; Ryan et al. 2003) and male evoked vocal response (Bosch et al. 2002, Bernal et al. 2007), we considered the possibility that the lack of enhanced response was due to the use of synthetic versions of the calls that lack potentially relevant properties for our particular question. The experiments with natural calls, however, confirmed our previous results

with synthetic calls. It is also possible that increased complexity is relevant in a specific context not evaluated by our study. Túngara frogs, for instance, make their mating decisions amid noisy environments with conspecific and heterospecific males calling. It is possible that multiple chucks are preferred when perceived against this naturally complex acoustic background. An effect of background noise on call discrimination has been detected in other species of frogs (Schwartz and Gerhardt 1998; Wollerman and Wiley 2002). Improved discrimination in the presence of noise, however, is unexpected in any auditory system (but see Schwartz and Gerhardt 1998). The potential role of background noise in discrimination of calls with greater call complexity has yet to be explored in female and male túngara frogs.

Here we tested calls that vary in the amount of sensory stimulation perceived by the receiver. Females of several species of anurans and insects prefer signals with traits of greater quantity such as calls that are longer, louder, and delivered at a faster rate (Ryan and Keddy-Hector 1992; Gerhardt and Huber 2002). Ryan and Keddy-Hector (1992) suggest that female preferences for these stimuli reflect a preference for greater sensory stimulation, and those preferences ultimately contribute to the evolution of complex signals. Our results suggest, however, that although males can produce signals of greater complexity, females do not appear to have driven the evolution of this phenotype. The lack of preferences in females for greater chuck number probably underlies the low frequency of calls with more than one chuck in nature. Whines with two or more chucks are about one tenth of the calls produced by calling males (Bernal et al. 2007). Moreover, the absence of female preference may also explain why males do not increase their

calling response to calls with several chucks. If females do not find calls with a greater number of chucks more attractive, there is no real threat of competition and males do not enjoy any benefits when increasing their vocal response to those calls types.

1.5. Acknowledgments

We are grateful to the assistants that helped us record the calls, analyze the recordings and performed female choice experiments. The Smithsonian Tropical Research Institute provided critical logistic support. The Autoridad Nacional del Ambiente de Panama provided the permits to perform this research. The frogs were marked following the Guidelines for the Use of Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR), available at http://www.asih.org/pubs/herpcoll.html. This work was funded by grant IBN 0078150 from the National Science Foundation.

Chapter 2: Sex differences in response to non-conspecific advertisement calls: Receiver permissiveness in male and female túngara frogs

Abstract. In many species males advertise to both male and female audiences. Given the asymmetry in fitness costs of recognition errors in response to mating signals for the sexes, usually higher for females than males, males are expected to be more permissive than females in their responses to signals. Few studies, however, have investigated such differences and there is no consensus on which sex is more permissive to signal variation. We examine the evoked vocal responses of male túngara frogs to fourteen species of heterospecific and ancestral male mating calls to evaluate the influence of call similarity and phylogenetic distance on their responses. We also compare male calling responses to female phonotactic responses to examine the propensity of response errors between the sexes. Recognition errors were higher for males than females as predicted by the different costs associated with recognition errors for each sex. Males responded to the calls of most species with mating calls, and produced aggressive calls in response to two other heterospecific/ancestral calls. The responses of males were explained by phylogenetic distance but not by overall call similarity. Similarly, females are more likely to exhibit phonotaxis to calls of species and ancestors that are more closely related. Therefore, evolutionary history has left a footprint on the brain of both sexes but the details seem to differ. We discuss proximate reasons underlying sexual differences in receiver permissiveness in túngara frogs and potential factors leading to their evolution.

In animal communication systems the same signal often targets multiple intended receivers. This is particularly common with mating signals where in many species males advertise to both male and female audiences (McGregor & Dabelsteen 1996; Searcy & Nowicki 2000; Gerhardt & Huber 2002). For example, male crickets, frogs, and birds often use the same acoustic signal to attract females and to repel males. Males and females are not, however, expected to have the same threshold for responding to the same signal, especially when these signals are associated with reproduction (Trivers 1972). The sexes are likely to differ in their response criteria according to their consequences of erroneous responses. A female basing a mate choice on the call of the wrong species, for example, could forfeit its reproductive investment for a substantial part of the breeding season, while a male calling in response to a heterospecific would bear less dire consequences, perhaps merely wasted time and effort.

2.1. Introduction

In spite of the potential differences between the sexes in their propensity for recognition errors, few studies have investigated such differences (Searcy et al. 1981a, b; Searcy & Brenowitz 1988; Cynx & Nottebohm 1992; Dabelsteen & Pedersen 1993; Vicario et al. 2001; Nelson & Soha 2004). These studies, which have been limited to songbirds, show that females and males can respond to signals in different ways. Whether the female or the male is the more discriminatory sex, however, is not consistent in the few species tested. In red-winged blackbirds (*Agelaius phoeniceus*) imitations of conspecific calls by a mockingbird (*Mimus polyglottos*) did not elicit courtship solicitation displays in

females (Searcy & Brenowitz 1988) while the vocal response of males was similar to both the imitation and normal song (Brenowitz 1982). Analogous results, in which females are more selective than males, were also found in response to conspecific red-winged blackbird songs modified by removing the final trill (Beletsky et al. 1980; Searcy & Brenowitz 1988), and unnatural temporal patterns of the conspecific song in swamp sparrows (*Melospiza georgiana;* Searcy et al. 1981a, b). In contrast, female blackbirds (*Turdus merula*) were less critical than males when responding to variation in song features such as the degree of frequency modulation of the song (Dabelsteen & Pederson 1993). The same is true in white-crowned sparrows (*Zonotrichia albicollis*) and zebra finches (*Taeniopygia guttata*). Female white-crowned sparrows were less selective than males when responding to songs containing a phrase from a foreign dialect (Nelson & Soha 2004), and similarly female zebra finches showed weaker discrimination than males in their responses to calls that advertise the sex of the signaler (Vicario et al. 2001).

In this study we investigate the responses of male túngara frogs, *Physalaemus pustulosus*, to non-conspecific signals and compare them to the results from previous published studies on the responses of conspecific females to the same stimuli (Ryan & Rand 1995, 1999). We examine the responses of males to signals that vary in call similarity and phylogenetic distance addressing the following issues: 1) male permissiveness in response to heterospecific signals, 2) the role of phylogenetic distance and call similarity in explaining these responses, and 3) sexual differences in responses to non-conspecific calls.

Physalaemus pustulosus *and its species group*

The túngara frog, *P. pustulosus*, is a small leptodactylid frog that is allopatric with other species in the genus *Physalaemus* throughout most of its range, with the exception of the llanos of Venezuela where it is sympatric with *P. enesefae* (La Marca 1992). As in most species of frogs, male túngara frogs produce advertisement calls to attract females and at the same time deter rivals. Because the túngara frogs we study in Panama are allopatric with close relatives, evaluating recognition of advertisement calls of other members of the genus by female and male túngara frogs allows us to investigate the extent of signal generalization and permissiveness excluding confounding factors associated with current selective pressures like character displacement (Ryan et al. 2003).

Túngara frogs mate in complex acoustic environments and their recognition of conspecific calls has probably been shaped by the costs associated with failing to respond to relevant signals and with responding to erroneous ones. For túngara frogs, as for most receivers, the main challenge is to differentiate signals from background noise, which can consist both of signals from individuals of different species and environmental disturbances. Thus, the criterion for responding to a stimulus in túngara frogs is balanced by the benefits of correct responses (responding when a signal has occurred) and correct rejections (not responding to background stimulation) and the costs of false alarms (responding to background stimulation) and missed detections (failing to respond when a signal occurred; Wiley 1994). As mentioned before, this trade-off is often different for each sex. Female túngara frogs responding to non-conspecific signals lose time and energy pursuing incorrect mates, and could forfeit their egg clutch if they do not find a

conspecific male on time. This is supported by the fact that females found at breeding sites often drop their eggs if left overnight without a male. On the other hand, males responding to incorrect signals would devote additional time and energy to calling, but would simultaneously increase their chances of attracting a mate. The trade-off further diverges between the sexes given the strongly male biased operational sex ratio in this species (Ryan 1985). The costs of missed detections are lower for females than males since females have multiple opportunities to mate while mating opportunities for males are rare.

The *Physalaemus pustulosus* species group consists of two monophyletic groups (Cannatella & Duellman 1984, Cannatella et al. 1998). One clade is found east of the Andes in northern South America and includes *P. freibergi*, *P. petersi* and *P. pustulosus.* A second clade consists of species found west of the Andes in Ecuador and Peru such as *P. caicai, P. coloradorum* and *P. pustulatus* (Fig. 1). In this study we used the advertisement calls of the species described in the *P. pustulosus* group by Cannatella & Duellman (1984) and included *P. caicai* and three additional outgroups: *P. enesefae*, *P. ephippifer* and sp. A. Species A is an undescribed species from the state of Roraima in northern Brazil (see Ryan & Rand 1995).

The advertisement calls of all species we used are frequency modulated whines (Fig. 2.1). In these species the fundamental frequency of the call is the dominant and sweeps from about 1000 to 500 Hz. In some species, males facultatively produce complex calls adding suffixes to the whines when they interact acoustically with other males. In *P. pustulosus*, males can add between 1-6 short, multi-harmonic components

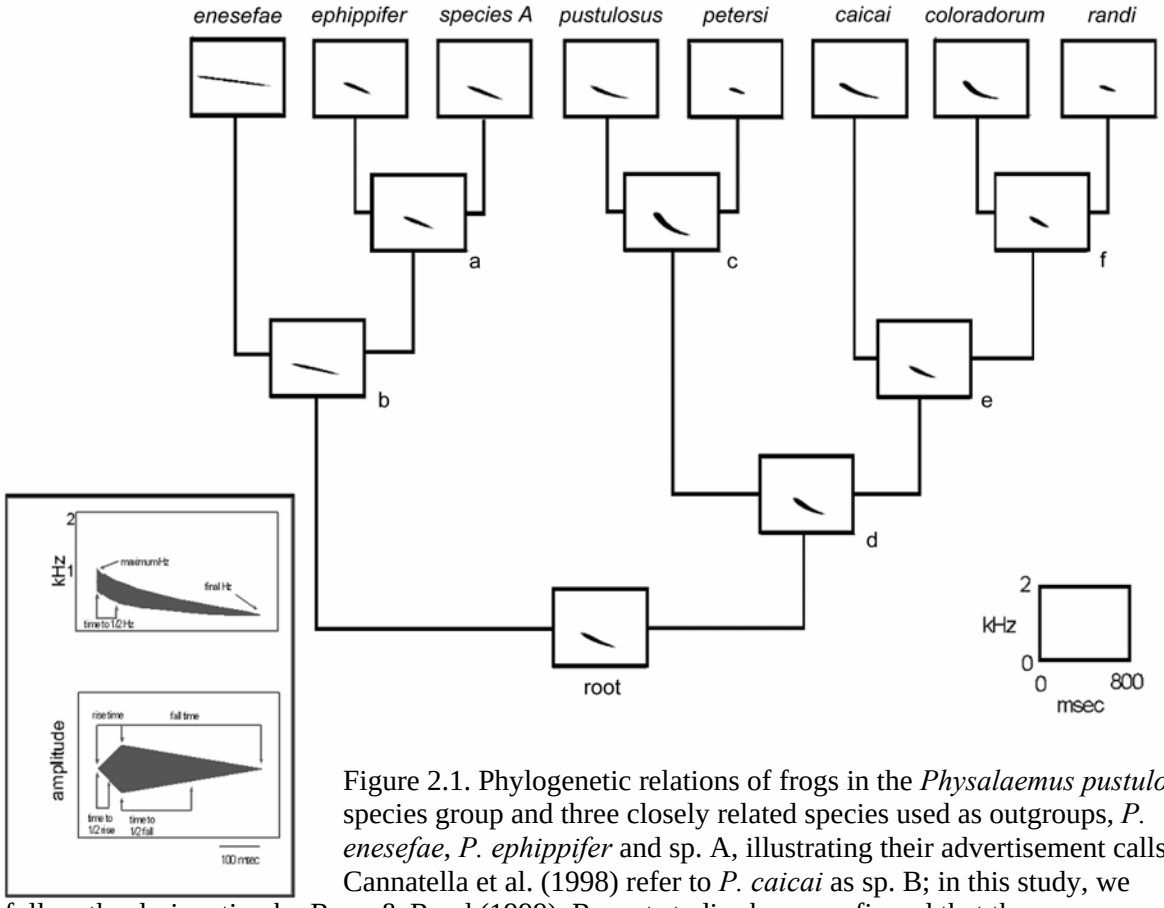

Figure 2.1. Phylogenetic relations of frogs in the *Physalaemus pustulosus* species group and three closely related species used as outgroups, *P. enesefae*, *P. ephippifer* and sp. A, illustrating their advertisement calls. Cannatella et al. (1998) refer to *P. caicai* as sp. B; in this study, we follow the designation by Ryan & Rand (1999). Recent studies have confirmed that there are several new species in western Ecuador and have also designated the population studied by Ryan & Rand (1995 & 1999), then known as *P. pustulatus,* as a new species, *P. randi* (Ron et al. 2004). Current studies, however, still support the monophyly of the species group and the presence of two clades (eastern vs. western of the Andes). Shown are sonograms of the synthetic advertisement calls of the extant species and estimations for the ancestral calls. The insert illustrates the call parameters measured to calculate species averages and synthesize test calls. Ancestral species are labeled in lowercase.

that are known as chucks (Ryan 1985). Chucks without whines, however, do not occur in nature. Complex calls are also known in some populations of *P. petersi*, the sister species of *P. pustulosus* (Boul & Ryan 2004). For the purposes of this study, however, we restricted the calls used as stimuli to only whines without including secondary components on the calls of any species.

2.2. Methods

We collected male *P. pustulosus* between May and August 2003 at breeding sites near the research facilities of the Smithsonian Tropical Research Institute in Gamboa (9°07.0'N, 79°41.9'W), Panama. Calling males were found at choruses and brought to the laboratory to be tested. After testing them, we measured the snout-vent length of the frogs, and gave them a unique toe-clip number to prevent them from being retested and to contribute to the long term data set on population demographics at this site. The frogs were marked following the Guidelines for the Use of Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR), available at http://www.asih.org/pubs/herpcoll.html. Túngara frogs have been toe-clipped in this population for two decades without any detectable negative effect. We then returned the frogs to the site where they were captured. The frogs were frequently recaptured in following nights but were not used in the experiments again.

Males responding to the experimental stimuli called antiphonally with the heterospecific/ancestral calls as they do when interacting acoustically with other males in nature (Greenfield & Rand 2000). We use the evoked vocal responses as an indicator of call recognition, a common approach in anurans (e.g., Ryan & Rand 1998; Bee 2003). Here we evaluate the calling response to non-conspecific calls in male túngara frogs and assume that if a signal elicits a response from a male, he is mistakenly identifying the stimulus to be a conspecific call. Therefore, responses to non-conspecific calls represent recognition errors, specifically false alarms.

Male Evoked Vocal Responses

We quantified the vocal response of males evoked by 14 calls of species of the same genus and estimates of ancestral calls. In the laboratory, we placed each male in a plastic bag; this type of plastic bag had been previously shown to be acoustically transparent by Ryan & Rand (1998). Each bag had sufficient standing water for the males to call, and we placed the bags inside individual acoustically-isolated chambers (30.5 cm x 46 cm x 30.5 cm) following Bosch et al. (2000a, 2002). The chambers were lined with sound absorbent material that attenuated sounds within the range of frequencies of the calls used in the experiment (500-1000 Hz). Each acoustic chamber contained a Radio Shack miniature microphone and a small, wide-frequency range speaker (Cambridge SoundWorks Inc., Ensemble IV). We placed males in the chambers the night they were collected and we tested them the next night, as previous experience suggested that males were more likely to respond if tested on the second night. No male spent more than two consecutive nights

in the laboratory. The chambers were in a well-ventilated room at typical calling temperatures, ca. 23-27°C. We maintained the males under a natural light-dark cycle and temperature regime. All males were tested between 1900 and 0200 hours.

We stimulated the males to call with a continuous tape of a high density túngara chorus recorded by A. S. Rand in Gamboa on 2 October 1990. Once a male began to call we initiated the playback experiments. Males were tested singly. Each test consisted of a set of five 60-second intervals: i) Control stimulus: white noise shaped with the amplitude envelope of the whine (0-10 kHz), ii) Silence, iii) Experimental stimulus: the heterospecific or ancestral call (see under Experimental Stimuli), iv) Silence, and v) Control stimulus (see also Ryan & Rand 1998; Bosch et al. 2000a). All calls and white noise were broadcast at a rate of one call every two seconds, a typical calling rate for túngara frogs (Ryan 1985). A total of 30 calls or whine-like noise bursts were presented in each 60-second intervals. Only cases in which males called during both control stimuli were included in the analysis to eliminate cases of no response due to lack of motivation. After each test, the male was required to call again or respond to the chorus before starting a new test. Each male was tested with all test stimuli or until he no longer responded (following Ryan & Rand 1998). We conducted a total of 14 experiments and in each one we tested ten males for a total of 140 male trials. A total of 39 males contributed to the data set.

We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 μPa) at 0.5 m measured by a GenRad sound pressure level meter model 1982. All experiments were presented in random order and recorded

with a Sony WM-D6 cassette recorder. We then digitized the tapes with CoolEdit 2000 (Syntrillum Software Corporation), at a sampling rate of 44.1 kHz and 16 bits/sample. Files were saved with coded names so measurements would be taken blind. In each 60-second interval of the experiment we counted the total number of whines, total number of chucks and maximum number of chucks appended to a single whine.

Experimental Stimuli

We examined the evoked vocal response to synthetic advertisements calls of five species of frogs in the *P. pustulosus* species group, three congenerics not in the species group, and calls reconstructed at the ancestral nodes (Ryan & Rand 1995; Fig. 2.1). Conspecific and heterospecific calls were recorded during previous studies and full details concerning such recordings are given there (Ryan & Rand 1993a; Ryan & Rand 1993b; Ryan & Rand 1999). Ancestral calls were estimated based on the calls of extant species (Ryan & Rand 1995; Ryan & Rand 1999). Variables for constructing the synthetic stimuli were based on mean values of a combination of the following eight spectral and temporal call parameters: maximum frequency, final frequency, duration, rise time, fall time, whine shape, rise shape, and fall shape. Phylogenetic relations among extant species and seven ancestral nodes were based on the most parsimonious tree topology determined from an analysis of several morphological characters, 27 allozymes, and 1200 base pairs of the 12S mitochondrial genome and its flanking regions, without including call characters in the analysis (Cannatella et al. 1998). Bootstrap estimates showed strong statistical support for all the nodes of the phylogeny (all $P < 0.05$). Estimates of the call characters

for the ancestral nodes were calculated from local squared-change parsimony and used to synthesize such calls. Ryan & Rand (1999) showed that although other models of evolution generate different estimations of the ancestral calls, these differences are not perceived by female *P. pustulosus*. More complete detail on the estimation of the calls and the model of evolution is found in Ryan & Rand (1999). We synthesized all stimuli using a program supplied by J. Schwartz (Pace University at Pleasantville, NY; sample rate 20 kHz and 8 bit).

Comparison between male and female responses

Ryan & Rand (1995, 1999) conducted phonotaxis experiments with female *P. pustulosus* to the same stimuli used in this study. We contrast our results on male evoked vocal response to the responses of female túngara frogs from the same population investigated in such study. Ryan & Rand (1995) quantified the number of false alarms in phonotaxis by females to the test calls paired with a white-noise stimulus as the one used as a control in this study (see Experimental Stimuli). In addition to those recognition experiments, the authors also presented conspecific calls paired with heterospecific calls in discrimination experiments. For the purpose of comparing female and male permissiveness to heterospecific/ancestral calls we used only the data on recognition experiments presented by Ryan & Rand (1995). In these experiments a response was scored when the female approached a speaker within 10 cm. A “no response” was noted if the female either remained motionless for 5 min after being released, stopped moving for 2 min at any time during the experiment or did not approach any speaker in 15 min. Tests to discern

absence of response due to lack of motivation rather than lack of attraction to the call were also performed. A complete description of the testing chamber and details of the protocol can be found in Ryan & Rand (1999).

Statistical Analysis

All statistical procedures were conducted using SYSTAT (Wilkinson 1991). To examine the recognition of heterospecific/ancestral calls in males, we contrasted their calling response to the experimental stimuli with their baseline calling behavior (average silence periods preceding and following the experimental stimuli). We characterized the overall response of males using the first component of a principal component analysis (PCA) combining the total number of whines, total number of chucks, and maximum number of chucks in a single call. The first component explained 83.43% of the variance and all the variables highly contributed to the analysis (Component loadings: number of whines=0.954, number of chucks=0.906, maximum chucks=0.879; eigenvalue =2.504). For each experimental stimulus we performed a separate Wilcoxon signed-ranked test using the scores from the PCA for each male during the presentation of the experimental stimuli and baseline calling.

Individual frogs varied in their absolute calling responses, thus to enable comparisons between individuals across stimuli we examined the strength of calling in response to each stimuli by averaging the calling response of the two controls to calculate the ratio of responses: experiment/(average control +1) (Bosch et al. 2000a, 2002). We then performed a PCA combining these ratios for the number of calls, number of chucks

and maximum number of chucks per call. We used Pearson correlation analyses to investigate the extent to which call similarity and phylogenetic distance explained male calling response to non-conspecific calls. These analyses are particularly interesting since call similarity and phylogenetic distance are not significantly correlated in this group of species (r=0.47, N=14, P=0.13, 95% CI=-0.08, 0.8). Our metric of call similarity was based on a PCA of the standardized call variables. We computed the Euclidean distances among calls based on the first three axes of variation from the PCA, which explained 87% of the variation in calls among species and nodes. Phylogenetic distance between *P. pustulosus* and the other extant species and the ancestral nodes was calculated based on the most parsimonious estimated changes in DNA base sequence (Ryan & Rand 1995).

To contrast the responses of the sexes to non-conspecific calls we converted the vocal response of males into a binary response equivalent to the one of females (i.e. response, no response). If a male called more during the presentation of the heterospecific/ancestral call than during the silent intervals before and after it, his behavior was scored as a response. A “no response” was scored when a male called less to the experimental stimuli than during silence. We compared the number of males and females that responded to each heterospecific/ancestral call using two-tailed Fisher Exact tests.

2.3. Results

Recognition errors in the form of false alarms were quite common. Most of the heterospecific calls we tested evoked higher vocal responses from *P. pustulosus* males

than spontaneous baseline levels (Fig. 2.2). In all but two cases (*P. enesefae* and ancestral node b), males had significantly higher calling PCA score during the presentation of the heterospecific call than during the silent periods before and after it. Males generally responded with both simple and complex calls to the experimental stimulus. Simple calls were produced at the beginning of the stimulus period, but often males escalated calling by adding chucks. In most cases, males increased the complexity of their calls in response to the heterospecific call. Although males can add up to six or seven chucks to a whine, the maximum call complexity exhibited during the experiments was only three chucks per call even though the total number of chucks summed over all calls varied considerably (15.09±10.85, range: 0-38 chucks).

Although males called less in response to *P. enesefae* and the ancestral node b, in those experiments they produced “mews”. Mews are aggressive calls that function in maintaining fixed spatial distances among chorusing males of this species (Ryan 1985). The aggressive call is clearly different from the whine; it is longer in duration and hashigher frequency and amplitude modulation than the advertisement call (Fig. 2.3).

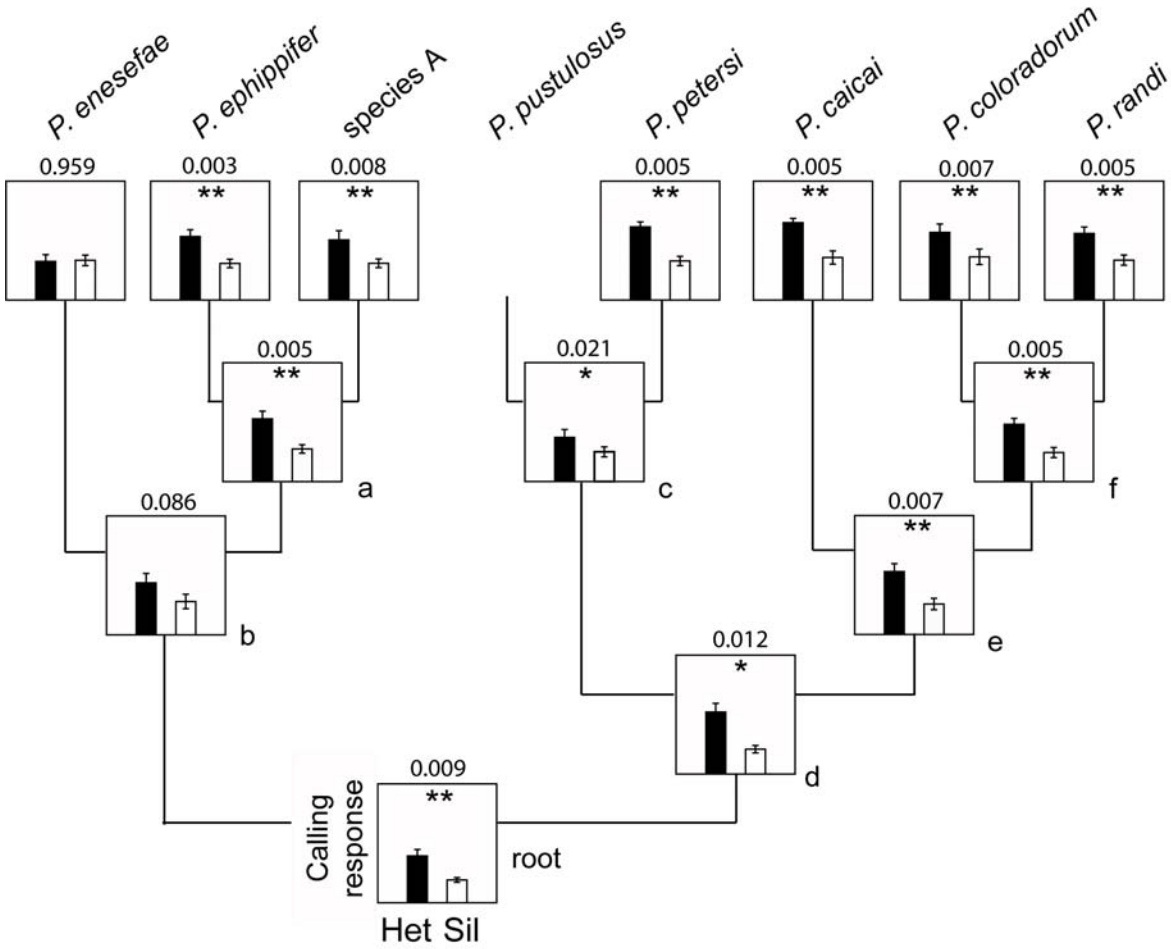

Figure 2.2. Responses of male túngara frogs, *P. pustulosus*, to "heterospecific" calls (Het). The calls were determined from species' means for the extant species and phylogenetic estimates for calls of the ancestral nodes. The responses of males (mean ± standard error) are based on Factor 1 of a PCA combining the number of whines, number of chucks and maximum chucks per call emitted in response to the heterospecific/ancestral call (black bars) versus spontaneous calling (white bars). See text for details about analysis. P-values of Wilcoxon signed-ranked test are shown on top of each graph, *P<0.05, ** P<0.001.

When exposed to *P. enesefae* calls, seven out of ten males produced mews, two did not call, and one produced whines after about 30 s of the experimental stimulus presentation. During the calls of ancestor node b, three males produced mews, three did not call, and four males produced whines. None of the other calls tested evoked mews.

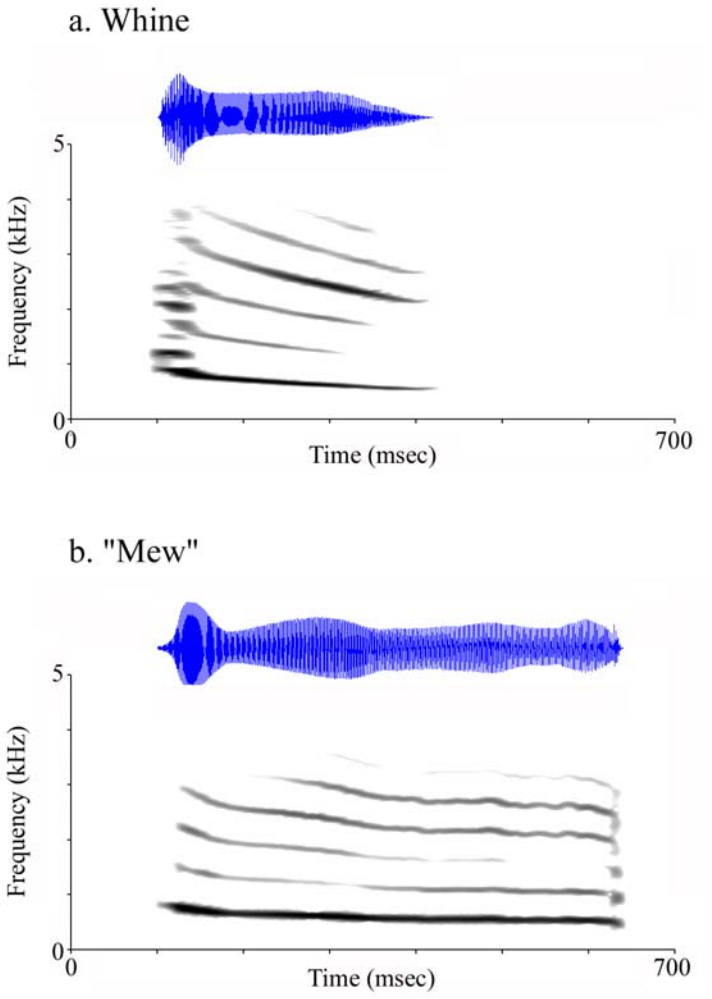

Figure 2.3. Advertisement call or whine (a), and aggressive call or "mew" (b) of túngara frogs. Sonograms are shown at the bottom and oscillograms on the top. The relative intensity of the calls is not represented proportionally in (a) compared to (b). The aggressive call is actually of much lower intensity than the whine.

There was no correlation between the overall call similarity of the test calls to the conspecifics call and the vocal response elicited by experimental stimuli (r=-0.341, N= 14, P=0.232; 95% CI=-0.738, 0.231). In contrast, phylogenetic distance significantly predicted calling response (r=-0.728, N=14, P=0.003; 95% CI=-0.907, -0.322). Multiple regression analysis is consistent with the results found in the correlations. Phylogenetic distance best predicts calling response (t=-3.123, N=14, P=0.010), while call similarity does not significantly explain any of the variation (t=-0.173, N=14, P=0.864).

Comparison between male and female responses

Males and females differed in their response to most of the calls we presented (Figure 2.4). In 9 out of 14 experimental stimuli the proportion of individuals that recognized the non-conspecific call was significantly different between the sexes. In addition, in all of those cases the proportion of males that responded to the heterospecific/ancestor call was higher than the proportion of females.

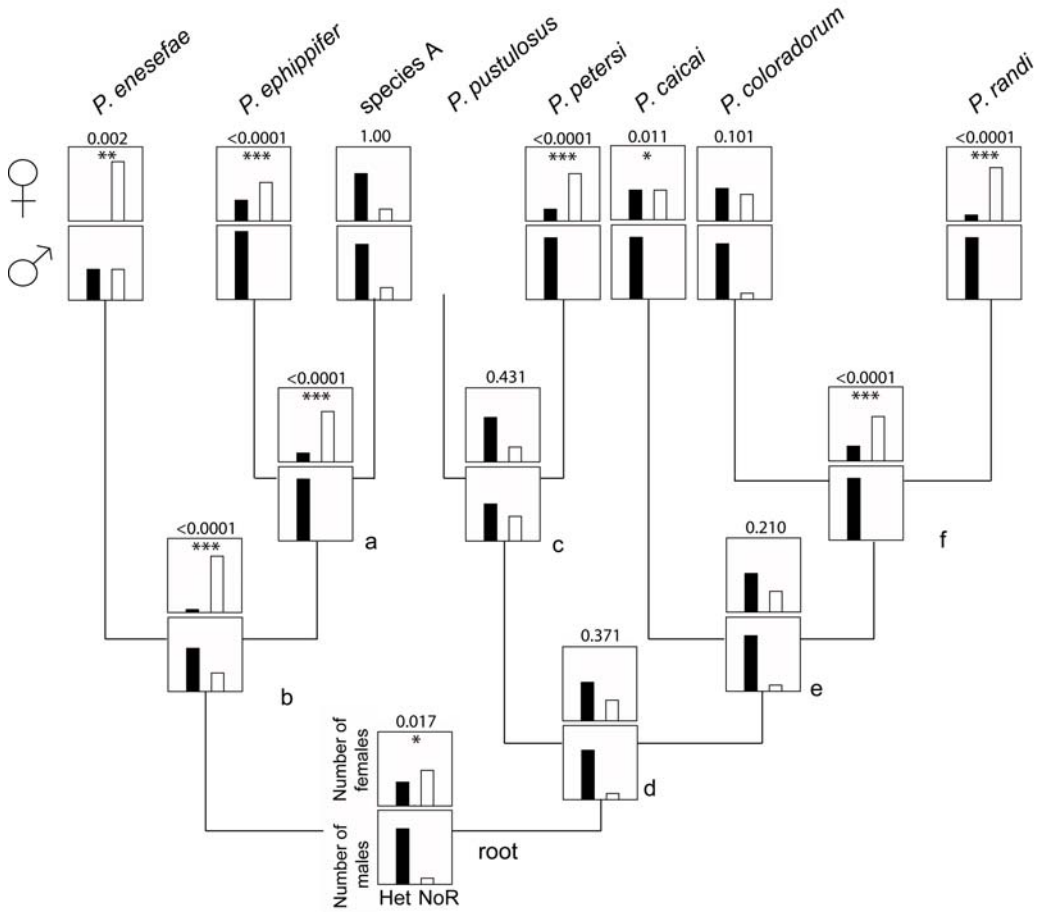

Figure 2.4. Responses of female (top) and male (bottom) *Physalaemus pustulosus* to the calls of species in the *P. pustulosus* species group, three closely related species, and calls estimated for the ancestral nodes. Responses to heterospecific/ancestral calls are in black bars (Het), and no responses are in white bars (NoR). Females' responses correspond to phonotaxis experiments conducted by Ryan & Rand (1995). Males' evoked vocal response is based on Factor 1 of a PCA combining calling response in number of calls and call complexity to score their behaviour as response/no response (see text for details). P-values of Fisher Exact test contrasting female and male responses are shown on top of each graph, *P<0.05, ** P<0.001, *** P<0.0001.

2.4. Discussion

We quantified recognition errors, specifically false alarms, in response to non-conspecific calls in male túngara frogs. To establish if the sexes differ in their responses as predicted by the differential cost of false alarms, we contrasted the responses of males to those of females to the same set of calls finding profound sexual differences.

Calling responses of male túngara frogs to non-conspecific advertisement calls

Male *P. pustulosus* increase their calling response to most of the calls we tested compared to their baseline calling during silence. In response to two calls (one heterospecific, one ancestral), the vocal response was not a mating call but an aggressive call. Given that males do not spontaneously produce aggressive calls, we consider the production of such calls an indication of call recognition, in this case a false alarm. Hence, males recognized all the calls we presented. They did not respond equally to all stimuli, however. Significant variation in male calling response was explained by phylogenetic distance but not by overall call similarity. The lack of relation between calling response and call similarity probably results from males weighting call characteristics in a way not captured in our measure of call similarity. We used a principal component analysis that accurately quantified call parameters, and is an appropriate technique to statistically distinguish calls, but it need not reflect the degree to which male túngara frogs perceive those same calls as different. Ryan & Rand (2003), for example, showed substantial differences between statistical variation in a population of mating calls compared to the calls' perceptual variation to female túngara frogs.

Recognition of such a broad range of stimuli as the one found in this study is unexpected. Even though several studies have investigated the vocal response of males to variation in specific call parameters (e.g. Walkowiak & Brzoska 1982; Schwartz & Wells 1984a; Allan & Simmons 1994; Penna et al. 1997), few have evaluated their responses to heterospecific calls. In a pioneering study, Capranica (1965) determined the calling response of male bullfrogs, *Rana catesbeiana,* in response to the calls of 34 species including nine species in the genus *Rana*, four of which are part of the *R. catesbeiana* species group (Hillis & Wilcox 2005). Male bullfrogs only called in response to conspecific calls. Capranica's results suggest high species specificity in the calling response of male bullfrogs. The difference in the results found by Capranica (1965) and our study may be due to the degree of similarity of the vocalizations used. The advertisement calls of all the *Physalaemus* we studied consist of frequency modulating whines similar to the one of *P. pustulosus* while the call of *R. catesbeiana* has two distinctive frequency peaks that are absent in the calls of other frogs of the same genus.

The general permissiveness in the response of male túngara frogs in Panama to other *Physalaemus* calls might also result from their being allopatric with all of their congeners. In contrast, several closely related species of *R. catesbeiana* are sympatric with this species in the eastern United States. One might expect sharpening of male call recognition in sympatry, just as one would expect reinforcement of female mating preferences. Evidence, however, suggests that the consequences of sympatry may be taxon specific. While in an Australian leptodactylid males suppress their vocal activity when exposed to calls of sympatric species (Littlejohn & Martin 1969), the opposite is

true in three species of neotropical treefrogs (Schwartz & Wells 1983a, 1983b, 1984a, 1984b, 1985).

Male *P. pustulosus* gave aggressive calls in response to *P. enesefae* and the ancestral node b call. In the laboratory males presented with this aggressive call in playback experiments produced aggressive calls in response, but often stopped calling immediately after the stimulus is broadcast. It thus appears that male túngara frogs perceived the calls of *P. enesefae* and node b as aggressive signals. This phenomenon may be the result of the long duration of the call of these test calls which resembles the mew. Schwartz & Wells (1984a, 1985) observed that males of *H. ebraccata* and *H. microcephala* increased the number of aggressive calls in response to high-intensity playbacks of both conspecific and heterospecific calls. These species occur at the same breeding sites and call in close proximity, thus they may have been favored to respond to each others' aggressive calls. Testing the calling responses of male túngara frogs from the llanos of Venezuela where they co-occur with *P. enesefae* could provide valuable insights.

Sexual differences in receiver permissiveness

Males made more recognition errors than females when responding to most of the non-conspecific calls. In spite of the differences between the sexes, all the heterospecific/ancestor calls recognized by females elicited only advertisement calls from males while the calls of species that elicited aggressive calls in males were not recognize by females.

Contrary to the results found for male calling response in this study, Ryan & Rand (1995, 1999) found that female recognition of the test calls was explained by overall call similarity as well as by phylogenetic distance. Thus calling males and females approaching a mate appear to weight signal variation differently. Our comparisons, however, reveal that although males and females disagree in how they respond to the different calls, their responses both show an effect of evolutionary history. Females are more likely to exhibit phonotaxis to calls of species and ancestors that are more closely related (Ryan & Rand 1995, 1999). Similarly, males call more in response to the calls of close relatives. Hence there is an effect of evolutionary history on response to advertisement calls in both male and female túngara frogs. Therefore, just as history has left a footprint on the females' brain, the same is generally true for males but the details seem to differ.

Differences in responses between the sexes could result from differences in the perception of signal variation, or in differences in how the same perceptual information influences decision making. In this mating system, as in many others, sexual signals elicit different tasks in the different sexes. Sexual differences in decision making could be either sex-specific independent of task, or task-specific independent of sex. For example, if females also called and males also exhibited phonotaxis we might find either that females are always more restrictive and less error prone to call variation whether hopping towards it or calling to it, or alternatively, that females are more restrictive in calls they approach but less restrictive in their vocal responses. The analogous thought experiment could be conducted with males, and actual experiments could, perhaps, be conducted in

duetting songbirds in which both female vocal response and courtship solicitation displays could be measured in response to the same set of signal variation.

The same confound of task and sex also occurs in other systems, such as the studies of Searcy & Brenowitz (1988) in red-winged blackbirds, Dabelsteen & Pedersen (1993) in blackbirds, and Searcy et al. (1981a, b) in swamp sparrows. At least two other studies, however, tested how males and females responded to signal variation using the same bioassay. Nelson & Soha (2004) measured calling responses of male and female white-crowned sparrows (*Zonotrichia albicollis*) to songs containing a phrase from a foreign dialect and showed that males were more discriminating. Similarly, Vicario et al. (2001) evaluated the calling responses of both sexes of zebra finches (*Taeniopygia guttata*) to calls that indicate the sex of the signaler and found that females showed weaker sex discrimination than males. A relevant consideration, however, is that in animals where females and males characteristically perform different behaviors in response to mating signals it is biologically meaningful to address their responses performing their sex-typical task.

Regardless of where the sexual difference arises, there are three hypotheses that predict them. These hypotheses have been proposed to explain differences between males and females in responses to bird song and can be extended to understand the same phenomenon in other systems of acoustic animals such as frogs. First, Dabelsteen & Pedersen (1988) suggested that if males and females differ in their habitats, they might experience different degrees of sound degradation and thus have evolved different responses to signals. Such a scenario is feasible in frogs (e.g., Witte et al. 2005). It seems

unlikely to us, however, that male and female túngara frogs responding to mating calls at the same breeding site experience different acoustic environments. Second, Kroodsma (1999) proposed that since male-male interactions lead to local song dialects and males would benefit from producing a common, widely distributed song to attract females over a wide area, males should be highly responsive to local song variants while females should attend to general, species-specific features of the call. Therefore, males are expected to be more selective than females. Our findings, however, are contrary to this prediction.

Third, the risk-of-investment hypothesis was initially proposed by Searcy & Brenowitz (1988) and later named by Dabelsteen & Pedersen (1993). As mentioned above, the consequences of false alarms and missed detections are more costly for females than for males, and thus females are predicted to be more coy. Another hypothesis, which to us seems an extension of this hypothesis, is the suggestion by Ratcliffe & Otter (1996) that females are under stronger selection to evaluate individual qualities of males. Female túngara frogs attend to spectral features of both the whine (Bosch et al. 2000b) and the chuck (Ryan 1980, 1985) of the mating call in a manner that result in females choosing larger males who then fertilize more eggs. Males also use spectral features of the conspecific whine; their calling behaviour escalates based on the frequency of their competitor's calls relative to their own call (Bosch et al. 2000a). Even though both sexes attend to call features that vary within the species, it is likely that males are not under such strong selection as females to ascertain species recognition.

Relaxed selective pressures on male calling responses could generate broader recognition functions while still enjoying within-species selectivity.

2.5. Conclusion

We compared recognition errors, specifically false alarms, between the sexes by comparing sexual responses to mating calls of a variety of non-conspecific signals. We found that male túngara frogs respond to an unexpectedly broad range of calls. Even though numerous studies have evaluated vocal behavior of male frogs to variation of various temporal and spectral parameters, few have examined variation outside the range of parameters of the conspecific call. In accord with the predictions based on the consequences of false alarms and missed detections, males were much more permissive in their response to signal variation than females. Several targets of selection could be responsible for such differences. They could result from differences between the sexes in perception, or from how the same information results in different decisions for sex-specific tasks.

2.6. Acknowledgements

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Chapter 3: Male evoked vocal response to heterospecific advertisement calls in *Physalaemus pustulosus*

Abstract. Little is known about how heterospecific calls influence behavioral responses in male anurans. This study focuses on the calling behavior of túngara frogs, *Physalaemus pustulosus,* to allopatric, congeneric calls. We examine the evoked vocal responses of male túngara frogs to the advertisement call of their own species and seven heterospecifics to evaluate their calling selectivity and response function beyond the range of conspecific signals. Using allopatric calls allows us not to confound current selection. Males show low selectivity responding to the playbacks of all the calls, they produced whines, added chucks to their calls and, in some cases, produced aggressive calls. The highest degree of response was elicited by the call of sp. B, a species in the *P. pustulosus* species group. Greater evoked vocal response to a heterospecific call than to the conspecific signal probably represents a response to supernormal stimulus. We also contrast the response function of males to the calls of their congenerics to previously published response functions of female túngara frogs to the same set of signals. Males' response function has a wider breadth and different shape than the one of females. This sexual difference in response functions represents different process of stimulus generalization probably due to different selective pressures on males and females.

3.1. Introduction

Social interactions in anurans are mediated mainly by acoustic signals. Frogs vocalize to attract mates, defend territories, and deter rivals (reviewed in Gerhardt and Huber 2002). These advertisement calls contain stereotyped species-specific characteristics that convey information on species identity. Frogs and toads are usually under strong selection to respond to calls of conspecifics and not to respond to calls of heterospecifics (but see Phelps et al. 2007). Response to a heterospecific call results in costs such as mismatings or displaying to the wrong rival. Thus, current behavioral responses and preferences for signals are assumed to be the product of selection to avoid such errors.

There are many cases in which animals show preferential responses to conspecific signals over heterospecific signals when the heterospecifics are allopatric (e.g. Coyne and Orr 1989; Crapon de Caprona and Ryan 1990; Nevo and Capranica 1985; Ryan and Rand 1995). Lack of response to allopatric heterospecifics can results from strong selection to recognize the conspecifics signal alone (Passmore 1981; Paterson 1985), or it can be an incidental consequence of past selection for conspecifics to discriminate against sympatric heterospecifics. In general, we would expect signal selectivity to be lower in response to allopatric heterospecifics. There are cases, however, in which females prefer exaggerated signals, and this may lead to preferences for signals outside the range of the conspecific call (Ryan and Keddy-Hector 1992). Preference for exaggerated or supernormal stimuli (Enquist and Arak 1993; Tinbergen 1953) may ultimately contribute to the evolution of complex signals.

There is little known about how heterospecific calls influence behavioral responses in male frogs. In playback experiments, male anurans of the few species investigated show dissimilar responses when presented with the advertisement calls produced by males of other species. For example, male bullfrogs (*Rana catesbeiana;* Capranica 1965*)* and the Southern toadlet (*Pseudophryne semimarmorata;* Littlejohn and Martin 1969) show high species-specificity when presented with calls of other species. In contrast, three sympatric and synchronic species of neotropical treefrogs (*Hyla ebraccata*, *H. phlebodes* and *H. microcephala*) do not show strong selectivity; they increase their calling behavior in response to their calls as well as those of heterospecific calls (Schwartz and Wells 1983a, b; 1984a, b; 1985). This disagreement in the modest literature on male calling behavior leads to questions about the selectivity of evoked vocal responses across anurans and the factors shaping them. Here we examine the vocal responses of male túngara frogs, *Physalaemus pustulosus*, to the calls of seven allopatric, congeneric species.

The calls of túngara frogs and their relatives used in our study are frequency modulated sweeps with a fundamental frequency that extends from about 1000 Hz to 500 Hz (Fig. 3.1). We used the calls of the species described in the *P. pustulosus* group by Cannatella et al. (1998) and three outgroup taxa: *P. enesefae*, *P. ephippifer* and sp. A. The latter is an undescribed species from the state of Roraima in northern Brazil. Túngara frogs are allopatric with other species in the genus *Physalaemus* throughout most of its range, with the exception of the llanos of Venezuela where it is sympatric with *P. enesefae* (La Marca 1992). As these experiments were conducted in Panama, the frogs

tested were allopatric with all congeners. This geographic distribution of the species in the genus *Physalaemus* allows us not to confound current selection pressures, such as character displacement, with historical biases.

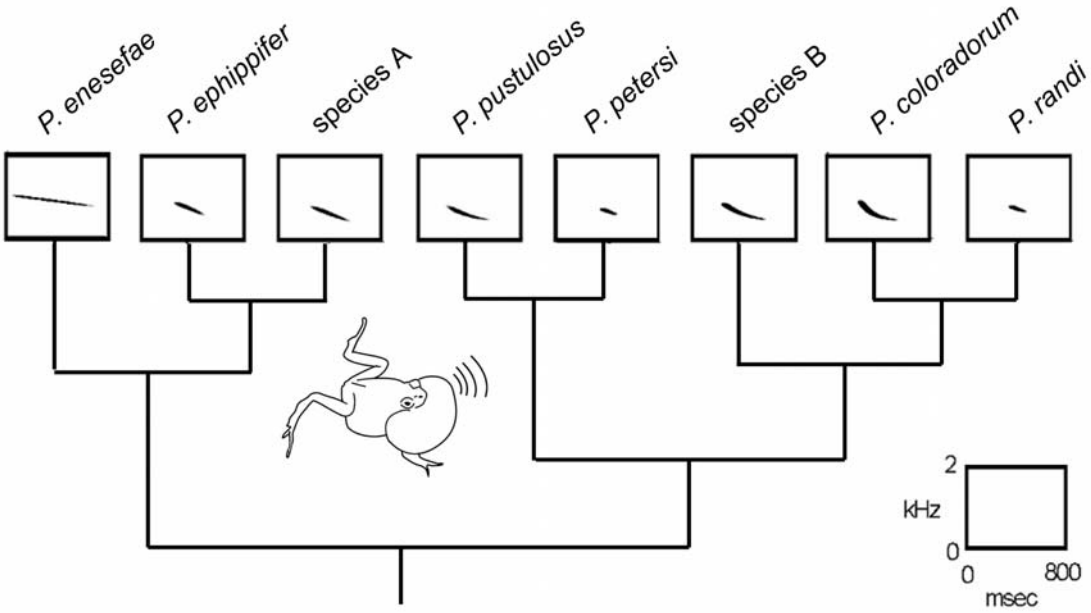

Figure 3.1. Advertisement calls of frogs in the *Physalaemus pustulosus* species group and three closely related species used as outgroups, *P. enesefae*, *P. ephippifer* and sp. A. Ryan and Rand (1999) refer to sp. B as *P. caicai*; in this study, we follow the designation by Cannatella et al. (1998) and Ron et al. 2006. Species A is an undescribed species from the state of Roraima in northern Brazil (see Ryan and Rand 1995).

Male túngara frogs recognize the mating calls of the congeneric species used in this study calling more to those calls than during spontaneous calling (Bernal et al. 2007). It is unclear, however, how their vocal response to heterospecific signals compares to the response to the conspecific call. Here we test the hypothesis that the conspecific call elicits the greatest vocal response, as expected if the signal has evolved to elicit maximum stimulation in the receiver. We also discuss the response function of males to

the calls of their congenerics in contrast with previously published response functions of female túngara frogs to the same set of heterospecific and conspecific signals.

3.2. Methods

We collected male *P. pustulosus* near the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07.0'N, 79°41.9'W). We found calling males at choruses and brought them to the laboratory. After testing them, we gave them a unique toe-clip to prevent retesting the same individuals. We then released the frogs at the breeding area where they were originally captured.

To quantify the vocal response of males in the laboratory we placed each male in an acoustically transparent plastic bag with sufficient standing water for the males to call (Ryan and Rand 1998). We placed the bags inside individual acoustically-isolated chambers (30.5 cm x 46 cm x 30.5 cm) following Bosch et al. (2000; 2002) and Bernal et al. (2007). These chambers are lined with sound absorbent material and each chamber contains a Radio Shack miniature microphone and a small, wide-frequency range speaker (Cambridge SoundWorks Inc., Ensemble IV). We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 μPa) at 0.5 m measured by a GenRad sound pressure level meter model 1982. The chambers are in a well-ventilated room at typical calling temperatures, ca. 23-27°C, and we maintained the males under a natural light-dark cycle and temperature regime.

We used a continuous tape of a high density túngara chorus recorded by A. S. Rand in Gamboa on 2 October 1990 to stimulate the males to call. Males were tested

singly as they began to call. Each test consist of a set of five 60-second intervals: i) Control stimulus: white noise shaped with the amplitude envelope of the whine (0-10 kHz), ii) Silence, iii) Experimental stimulus: the conspecific or heterospecific call (see under Experimental Stimuli), iv) Silence, and v) Control stimulus (see also Bernal et al. 2007, Bosch et al. 2000, Ryan and Rand 1998). We broadcast all calls and white noise at a typical calling rate for túngara frogs of one call every two seconds (Ryan 1985). All experiments were presented in random order and recorded with a Sony WM-D6 cassette recorder. We then digitized the tapes using CoolEdit 2000 (Syntrillum Software Corporation), at 44.1 kHz sampling rate and 16 bits/sample. In each 60-second interval of the experiment we measured total number of whines, total number of chucks and maximum number of chucks in a single call. In the analysis we only included cases in which males called during both control stimuli to discard cases of no response due to lack of motivation.

Experimental Stimuli

We examined the vocal response to synthetic advertisements calls of *P. pustulosus*, four additional species of frogs in the *P. pustulosus* species group, and three congenerics not in this group (Ryan and Rand 1995; 1999). The calls of all species were recorded during previous studies and complete details about such recordings are given there (Ryan and Rand 1993a, b; 1999). Variables for constructing the synthetic stimuli were based on mean values of a combination of the following eight spectral and temporal call parameters: maximum frequency, final frequency, duration, rise time, fall time, whine

shape, rise shape, and fall shape. All stimuli were synthesized using a program supplied by J. Schwartz (Pace University at Pleasantville, NY). Complete details on the estimation of the calls and the synthesis of the stimuli are presented in Ryan and Rand (1999).

In some species of *Physalaemus*, males facultatively add suffixes to the whines when they interact acoustically with other males. In *P. pustulosus*, males may add 1-7 short, broad band suffixes known as chucks (Ryan 1985). Suffixes are also produced in some populations of *P. petersi*, the sister species of *P. pustulosus* (Boul and Ryan 2004). To avoid confounding factors of increased responses to calls with suffixes to the calls of some species we restricted the calls used as stimuli to only whines without including secondary components on the calls of any species.

Statistical Analysis

Given that individual frogs vary in their absolute calling responses, to enable comparisons between individuals across stimuli we examined the strength of calling in response to each stimulus by averaging the calling response of the two controls to calculate the ratio of responses: experiment/(average control +1) (following Bosch et al. 2000, 2002). Using these ratios we asked whether male vocal response differs in their response to heterospecific calls versus conspecific calls in the following parameters: (i) call rate, (ii) total number of chucks, and (iii) maximum number of chucks added to a single call. To compare the calling behavior to heterospecific calls to the one elicited by the conspecific call we used Mann-Whitney tests using SYSTAT (Wilkinson 1991). To

account for multiple comparisons we used a sequential Bonferroni method correction (Rice 1989).

3.3. Results

Male túngara frogs called during the presentation of all of the heterospecific calls we used in the experiment. Males responded to the playbacks producing whines, whines with chucks, and in some cases aggressive calls. The evoked vocal response elicited from *P. pustulosus* males was specific to the stimulus. Aggressive calls were produced in response only to the call of *P. enesefae*. Seven out of ten males produced aggressive calls, and the remaining three males did not call at all during the presentation of *P. enesefae* or called after about 50 sec of presentation of such call. Aggressive calls were not elicited by the calls of any other heterospecific species.

There was significant variation in number of calls, total number of chucks, and maximum number of chucks appended to a single call between experimental stimuli (Fig. 3.2; Kruskal-Wallis: number of calls H=25. 815, P= 0.001; total chucks H=21.527, P=0.003; maximum chucks per call H=0.016, P=0.016). The greatest calling response, however, was not produced when the males were exposed to the conspecific call. Males produced more whines in response to the call of sp. B than to the average call of their own species (Fig. 3.2a; Mann-Whitney test: U=87.0, N_1=10, N_2=10, P=0.005). In contrast, the call of *P. enesefae* elicited fewer total chucks and tended to trigger production of fewer calls and fewer chucks per call than the conspecific call (Fig. 3.2b

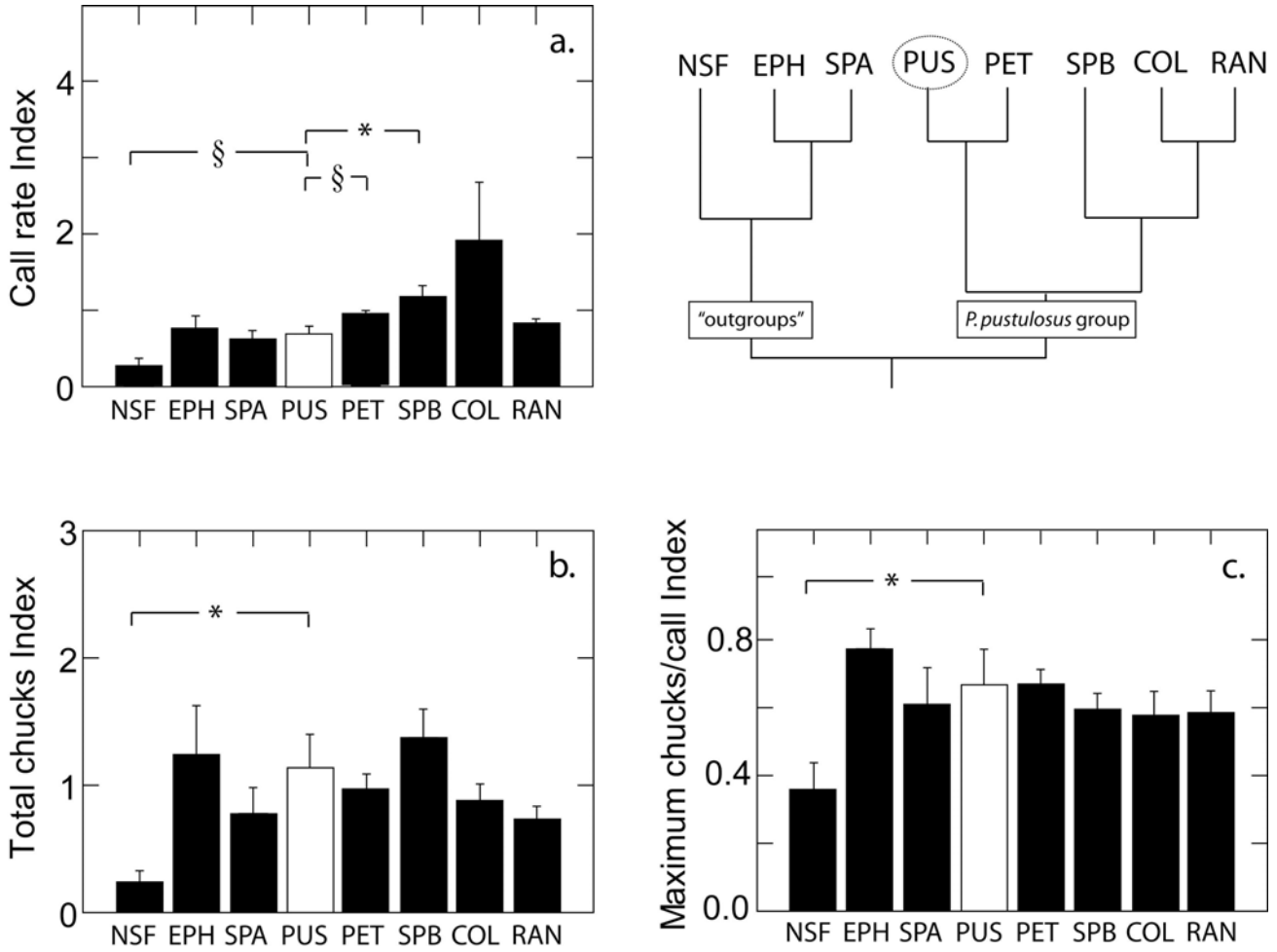

Figure 2. Calling responses of male *P. pustulosus* to playback experiments of heterospecific (black) and conspecific (white) calls. Response Indexes are calculated as the response to experimental stimulus divided by the average response in the controls +1. Mean ± SE are shown. On the upper right corner, a phylogenetic tree illustrates the most parsimonious hypothesis for the relationships among the species used in the experiments. NSF, *P. enesefae*; EPH, *P. ephippifer*; SPA, Sp. A; PUS, *P. pustulosus*; PET, *P. petersi*; SPB, sp. B; COL, *P. coloradorum*; RAN, *P. randi*. * denotes statistical significance, § denotes $P<0.05$ but not significant after the sequential Bonferroni correction.

and c; Mann-Whitney test: number of chucks, U=12.5, N_1=10, N_2=10, P=0.005; number of calls, U=21.5, N_1=10, N_2=10, P=0.031; maximum chucks per call, U=19.5, N_1=10, N_2=10, P=0.018). The call of *P. petersi*, the sister species of *P. pustulosus*, tended to increase call repetition rate in comparison to the conspecific call. The increase, however, is not significant after sequential Bonferroni corrections. Other comparisons of the strength of the evoked vocal response between the call of *P. pustulosus* and its congenerics species were not significantly different from the response to the conspecific call.

Although call rate increases without a parallel increase in call complexity in response to some experimental stimuli (e.g. sp B), generally the number of calls elicited per minute and the number of chucks appended to the calls are highly correlated (Pearson correlation, r=0.876, $P <$ 0.001). Thus stimuli that tend to increase call rate usually also elicit a parallel increase in call complexity.

3.4. Discussion

Results of our behavioral experiments show that the evoked vocal responses of túngara frogs, *P. pustulosus*, have low selectivity. Male túngara frogs called back in response to a wide range of heterospecific calls and in most cases, their response was equivalent or higher than the response to the conspecific call. The great responsiveness of male túngara frogs in Panama to the calls of other *Physalaemus* might be a consequence of relatively high similarity of the calls of the congenerics to the conspecific call. This, in turn, might be due to túngara frogs being allopatric with most species tested. An interaction of call

similarity and geographic distribution is also possible. Close relatives in allopatry, for instance, often have more similar calls than close relatives in sympatry. Such a trend has been reported in tropical treefrogs (Duellman and Pyles 1983).

As stated above, the calls of species in the *P. pustulosus* group and the outgroups all consist of frequency modulated whines within a similar range of frequencies (Fig. 3.1). It remains for the receivers, however, to discern whether or not signals are sufficiently different. In our study, males responded to most heterospecific calls similarly. From the perspective of female túngara frogs, the other intended receiver of these signals, this set of heterospecific calls is not homogeneous. Females expose to the same calls only respond to a minority of the calls, and prefer the conspecific call in all cases (Ryan and Rand 1995; Ryan et al. 2003) Thus these call differences are not meaningful to males while they are meaningful to females. This could be because only females, and not males, can perceive the acoustic differences, or that both sexes can perceive them, but only females take them into account in their responses.

High responsiveness of evoked vocal responses could also arise from túngara frogs being allopatric with all of their congeners, independent or in conjunction with the calls being fairly similar to one another. In Panama there are no other species of *Physalaemus* and the calls of the sympatric species are fairly different from the call of túngara frogs (Ibañez et al. 1997). Analogous to reinforcement of female mating preferences, sharpening of male call responses in sympatry could also take place. Consistent with this hypothesis, calls of sympatric species do not elicit vocal responses of male bullfrogs (Capranica 1965), and males of the Southern toadlet suppress their vocal

activity when exposed to calls of a sympatric species (Littlejohn and Martin 1969). Contrary to this prediction, however, small neotropical treefrogs (*Hyla ebraccata*, *H. phlebodes* and *H. microcephala*) increase their calling activity in response to the calls of the each other (Schwartz and Wells 1983a, b; 1984a, b; 1985). If males of different species compete for calling sites, as may be the case in these three synchronic small treefrogs, it is probably advantageous to call not only to deter conspecific rivals but also to avoid intrusions from males of other species. Relaxed selection pressures due to their allopatric distribution with congeneric species combined with relatively high call similarity are probably both important factors underlying the calling behavior of túngara frogs to the calls of other *Physalaemus*.

Playbacks using heterospecific calls typically elicited advertisement calls from túngara frog males. Aggressive calls were produced in a small proportion of the tests and were only elicited in response to the call of *P. enesefae*. The call of this species resembles the aggressive call of túngara frogs in its long duration, and is probably perceived as an aggressive signal. Males of *H. ebraccata* and *H. microcephala* also produce aggressive calls in response to heterospecific calls (Schwartz and Wells 1984a; 1985). In contrast to *P. pustulosus* and *P. enesefae*, these treefrogs call in close proximity at the same breeding sites. *Physalaemus pustulosus* and *P. enesefae* do call in close proximity in some sites in the llanos of Venezuela, this interaction has not been studies and it least in the field túngara frogs did not commonly produce aggressive calls in response to *P. enesefae* calls (MJR, personal observation). Evoked vocal calling of túngara frogs in this area warrants some consideration.

The greatest calling response was not elicited when male túngara frogs were exposed to the conspecific call. Males significantly increased call repetition rate, but not total number of chucks or maximum chucks per call, in response to the call of sp. B relative to the call of their own species. The call of sp. B is extremely similar to the túngara frog call. Of note, the call of sp. B is one of the few congeneric calls that female túngara frogs recognize (Ryan and Rand 1995). In a multiple dimensional scaling analysis, which explained 79% of the variation among call differences, the call of sp. B lies just outside a cloud formed by calls of 50 males from the test population (Ryan et al. 2003). The calls of sp. B and *P. pustulosus* differ slightly in all the variables measured, thus no single call parameter is a candidate for triggering an increase in response. This is further complicated by the fact that several call variables are correlated with each other. Recognizing these concerns, it is interesting to note that the average rise time is the trait that strongly differs between the calls of these two species being about four times longer in the call of sp. B than in the conspecific call (sp. B = 105.10 msec, *P. pustulosus* = 24.03 msec). This is also a trait that loads heavily in a PCA of call variation in this set of species (Ryan and Rand 2001). But in phonotaxis tests, female responses are not best described by call parameters that discriminate among species (Ryan and Rand 2001). Thus in the male responses, rise time could be acting as a supernormal stimulus, but further studies evaluating responses to variation in rise time in isolation are necessary to confirm this hypothesis.

Regardless of which call variable is involved in this process, greater evoked vocal response to a heterospecific call than to the conspecific signal probably represents a

response to a supernormal stimulus. In several species of anurans females prefer calls with traits of greater quantity (i.e. longer, higher intensity, more complex calls; reviewed in Ryan and Keddy-Hector 1992). It is also common that females prefer calls with traits outside the range of natural variation of the conspecific call (Gerhardt 1991; Gerhardt and Hubber 2002). Stronger calling response to exaggerated stimuli is therefore to be expected in males as well. Whether male frogs, in general, also respond more strongly to traits of greater quantity is unclear, however. Simmons (2004) investigated the calling response of male bullfrogs to stimuli with supernormal call durations finding no preference for those calls. Thus we report here a potential case of preference for supernormal stimulus that parallels this phenomenon typically described in female but not male anurans.

The low selectivity, combined with the highest response to a heterospecific call, raises questions about the shape of the calling response function for variation across the range of congeneric signals. Moreover, how does the response function of males compares to the one of females? Ryan and Rand (1993a, b, c) proposed a hypothetical set of female preference functions, *a* through *e*, shown in Fig. 3.3a. All these proposed functions would result in preference for conspecific over heterospecific signals reflecting the behavior of females. Based on their results, they further suggested that female túngara frogs show a preference function like *d*. This function illustrates the behavior of females of this species: they generate selection on conspecific signals, prefer signals of their own species over heterospecific ones, and respond only to some heterospecific signals. The question, then, is how does the response function of males compare to the one of

females? Functions *a′*, *d*, and *e′* illustrated in Fig. 3.3b result in recognition of heterospecific signals that could resemble the responses of males. Function *a′* would generate low selectivity but contrary to our findings would result in homogeneous behavior to heterospecific calls. Our results suggest that males' response function has a wider breadth than the one of females and a different shape resembling function *e′* instead of *d*. Male túngara frogs, for instance, show a higher call rate to sp. B than to the conspecific signal while producing few chucks in response to *P. enesefae*. Moreover, from previous studies we know that even though male calling response in this species escalates based on the frequency of their competitor's calls relative to their own call, there is no net selection on conspecific signals (Bosch et al. 2000). The difference in response functions between males and females represents different processes of stimulus generalization probably due to different selective pressures. It is likely that males are not under such strong selection as females to shape response selectivity (Bernal et al. 2007; Searcy and Brenowitz 1988). Relaxed selective pressures on male calling responses probably generated broader recognition functions.

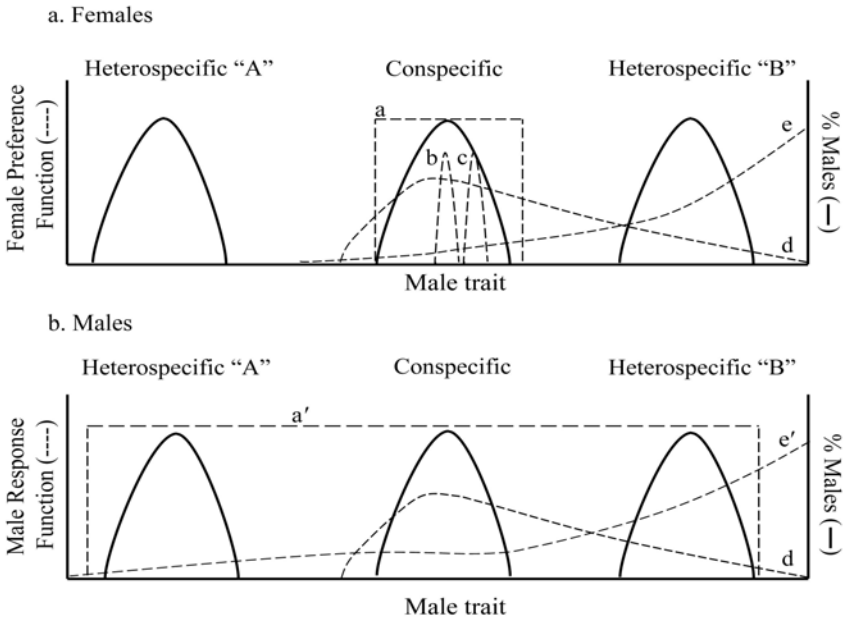

Figure 3. Hypothetical interactions between variation in male call traits and female (a) and male (b) preference functions. The solid lines show the distribution of variation in male traits for species "A" and species "B". Preferences functions in dashed line are showed for individuals of species "A". Adapted from Ryan and Rand (1993c)

3.5. Acknowledgements

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Chapter 4: Sexual differences in the behavioral response of túngara frogs, *Physalaemus pustulosus*, to cues associated with increased predation risk

Abstract. Engaging in mating behaviors usually increases exposure to predators for both males and females. Anti-predator strategies during reproduction may have important fitness consequences for prey. Previous studies have shown that individuals of several species adjust their reproductive behavior according to their assessment of predation risk, but few studies have explored potential sexual differences in these strategies. In this study, we investigate whether the acoustic cues associated with predatory attacks or acoustic cues associated with predators themselves affect the mating behavior of female and male túngara frogs, *Physalaemus pustulosus*. We compared the responses of females approaching a mate and those of calling males when exposed to mating calls associated with sounds representing increased hazard. When presented with mating calls that differed only in whether or not they were followed by a predation-related sound, females preferentially approached the call alone. In contrast, calling males showed greater vocal response to calls associated with increased risk than to a call by itself. We found significant differences in the responses of females and males to several sounds associated with increased hazard. Females behaved more cautiously than males suggesting that the sexes balance the risk of predation and the cost of cautious mating strategies differently.

4.1. Introduction

In most species engaging in mating behaviors entails high risks for both males and females. Advertising has been long recognized as a hazardous activity since Darwin acknowledged the dangers males undertake when displaying to attract females (Darwin 1859). Searching for a mate, however, has only recently been considered a risky behavior (reviewed in Lima & Dill 1990). Females navigating breeding areas in search of a mate are vulnerable to opportunistic predators attracted by signaling males and may be even more susceptible to predation than males themselves (Dill et al. 1999; Godin & Briggs 1996; Gong & Gibson 1996; Hedrick & Dill 1993; Pocklington & Dill 1995). In decorated crickets, for instance, Mediterranean house geckos localize burrows of calling males but cannot reach the cricket inside, thus they wait for approaching females also attracted to the calls of the male (Sakaluk & Bellwood 1984). In situations like this, selection may act on females to reduce predation-related costs. Consistently, there is increasing empirical evidence that susceptibility to predation affects female mating behavior in invertebrates, fish, birds and mammals (Gibson and Bachman 1992; Wilson et al. 1994; Gong and Gibson 1996; Johnson and Basolo 2003; Su and Li 2006). While dangers to males have been investigated, and recent evidence finds dangers to females too, so far there has been almost no attention paid to differences in male and female response to predation risk (Magurran and Nowak 1991; Su and Li 2006).

Given the differences in mating opportunities of males and females (Trivers 1972), and that anti-predator behaviors result in reduced mating activity, sexual differences in response to predation risk are expected. Females usually have multiple chances to mate

and should be under strong selection to reduce their susceptibility to predation in hazardous situations. For males, in contrast, the cost of missing an opportunity to obtain a female is high, and they are expected to incur higher predation risk to attract mates (Lima & Dill 1990; Magnhagen 1991).

In this study we investigate whether the acoustic cues of predatory attacks or the acoustic cues of predators themselves affect the mating behavior of female and male túngara frogs, *Physalaemus pustulosus*. Several predators attack and consume túngara frogs at the breeding ponds, including: frog-eating bats (Tuttle & Ryan 1981), four-eyed opossums (Tuttle et al. 1982), South American bullfrogs (Ryan et al. 1981), and large crabs (Ryan et al. 1981). Female and male túngara frogs use an array of strategies to avoid predation during mating. Females, for instance, are more likely to choose a mate and more likely to choose distant callers in the dark, when predators cannot see them, than under dim light (Rand et al. 1997). Similarly, males cease calling and may dive beneath the water's surface in response to the visual threat of an approaching frog-eating bat (Tuttle et al. 1982). Besides visual cues, male túngara frogs also use calls of non-conspecific frogs to assess predation risk and decide when to resume calling after a disturbance (Phelps et al. 2007). Here we ask whether female and male túngara frogs listen to other non-conspecific sounds to avoid predation, specifically the sounds produced by approaching predators or the sounds produced during predatory attacks on nearby frogs. We investigate the responses of females approaching mates and the calling response of males to mating signals in the presence and absence of sounds associated with increased predation risk.

4.2. Methods

We collected túngara frogs in the areas surrounding the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07.0'N, 79°41.9'W). All frogs were found at choruses between 1900 and 2200 h, brought to the laboratory where they were tested, and subsequently returned to the original site where they were found. Prior to returning the frogs, we gave them a unique toe-clip number to prevent them from being retested.

Acoustic stimuli

Male túngara frogs produce whine-like mating calls whose fundamental frequency sweeps from 1000 to 500 Hz in 300 msec. Based on the mean values of the parameters of the calls in the population we synthesized an average whine by shaping sine waves using the software developed by J. Schwartz (Pace University at Pleasantville, NY; sample rate 20 kHz and 8 bit). Mean values were calculated based on the calls from 50 males recorded in July 1996 with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003).

To simulate increased predation risk we used two kinds of experimental stimuli: i) sounds that indicate the proximity of potential predators, such as the advertisement call of the South American bullfrogs, *Leptodactylus pentadactylus*, or the sound produced by the

wings of a frog-eating bat in flight (Fig. 4.1); ii) sounds that indicate predatory attacks such as the sound of a quick movement in the water, hereafter a “splash”, and the sound of movement in leaf litter, or a “rustle” (Fig. 4.2). A splash simulates an attack by a bat or opossum that suddenly tries to catch a frog calling on water, as túngara frogs do in nature. This kind of attack also generates water borne cues, but for the purpose of this study we focus only on acoustic cues. A rustle imitates a terrestrial predator such as an opossum walking through leaves approaching the edge of the pond. Given the transient nature of sounds such as the splash and the rustle in the wild, we recorded these stimuli at the breeding ponds imitating events such as the ones described above, a predator attacking a frog calling on water or approaching the edge of the pond. We used a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone to record those sounds. The sound of the approaching bat was recorded by Rachel A. Page. A frog-eating bat, *Trachops cirrhosus*, was held by hand so it would start flapping its wings, and after several flaps the bat was released. The sound produced as the bat flew towards a Sennheiser ME-66 shotgun microphone was recorded using a WM-D6C Sony Tape recorder. A total of five individuals were recorded and the recordings with the least background noise were chosen to be used in the experiments. The advertisement call of *L. pentadactylus* was recorded in the Panama Canal area, a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone, following standard procedures (Heyer 1994).

We appended the sounds that simulated predators or attacks to a synthetic whine. The duration of the splash, the rustle and the sound of the bat in flight were standardized to 600 msec, appropriate duration to simulate the desired effect and avoid overlapping

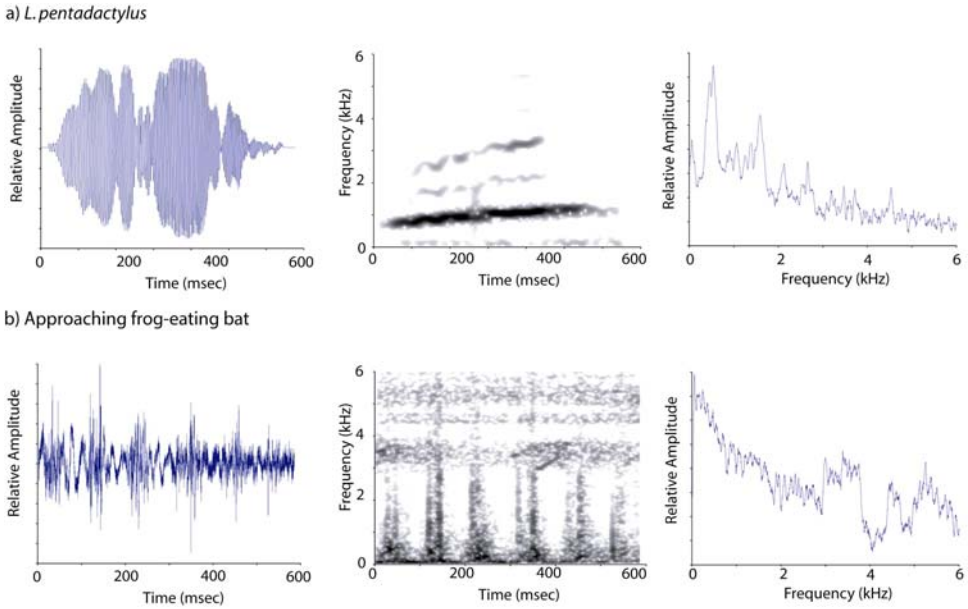

Figure 4.1. Oscilogram (left), spectrogram (center) and frequency spectrum (right) of the experimental sounds associated with proximity of potential predators. (a) Mating call of the South American Bullfrog, *Leptodactylus pentadactylus*. (b) Sound produced by the wing-beats of an approaching frog-eating bat, *Trachops cirrhosus*.

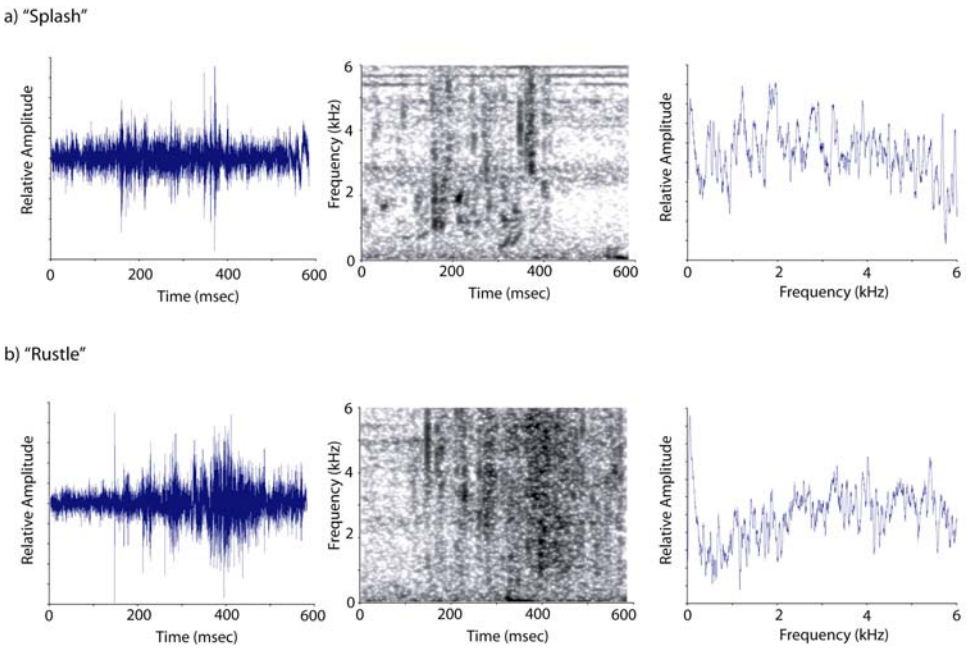

Figure 4.2. Oscilogram (left), spectrogram (center) and frequency spectrum (right) of the experimental sounds associated with predatory attacks. (a) "Splash", sound produced by a quick movement into the water. (b) "Rustle" sound produced by movement of leaf litter.

with the stimulus broadcast from another speaker in the tests with females where the stimuli were presented antiphonally from different speakers (see below). The total duration of each stimulus, whine plus threat sound, was 969 msec. The calls with the experimental sounds were broadcast at a rate of one call every two seconds, a typical calling rate for túngara frogs (Ryan 1985). To avoid acoustic masking, we did not broadcast the experimental stimuli at the same time as the whine. Playing the stimuli following the whine is a conservative measurement given that non-conspecific sounds appended to the call can increase call attractiveness to females (Ryan and Rand 1993), and therefore should bias the results in the direction contrary to our prediction.

To explore whether the responses of the frogs were the effect of any relatively long, loud sound appended to the call, we also used a modified natural component of the mating call in addition to the predatory stimuli described above. Male túngara frogs facultatively add burst-like secondary components to the whine, called chucks. These suffixes are shorter than the whine, about 40 msec in duration, and increase both the attractiveness of the call to females and the calling response of males (Rand & Ryan 1981; Ryan 1985). Using the mean parameters of the chuck in the population and the methods described above, we synthesized a call component identical to a natural chuck in all features but duration. We produced a synthetic chuck of equal duration to the experimental stimuli, hereafter “long chuck” (Fig. 4.3).

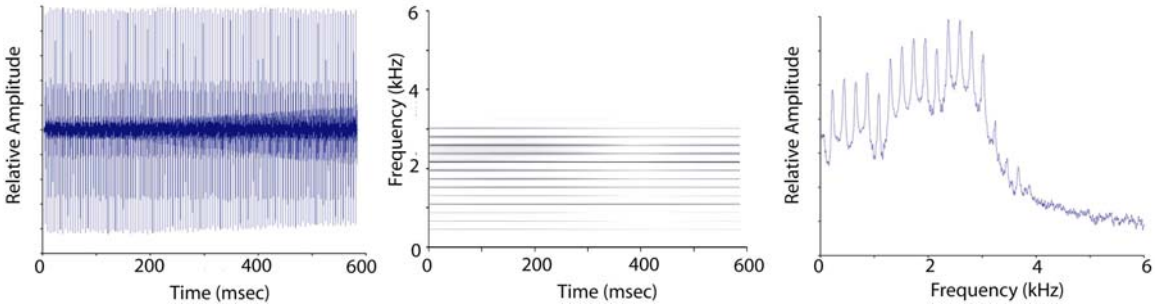

Figure 3. Oscilogram (left), spectrogram (center) and frequency spectrum (right) of the "long chuck" used in the experiments as a control stimulus (see text for details).

Females - phonotaxis

In 2002 and 2003, between May and August, we performed standard two-speaker phonotaxis tests offering females a choice between identical mating calls with and without one of the experimental stimuli. Each female was placed under a funnel in the center of a 1.8 m x 2.7 m sound attenuation chamber (Acoustic Systems, Austin, TX). The stimuli were broadcast for 3 min before remotely removing the funnel allowing the female to freely move in the arena. We broadcast the test stimuli antiphonally from speakers in the center of walls opposite one another such that the peak amplitude of the whine of each test call at the center of the arena was 82 dB SPL (re. 20 µPa). A choice was scored when the female approached any of the speakers within 10 cm. No choice was scored if a female stayed at the release point without moving for 5 min, did not move for 2 min, or spent more than 15 min roaming the arena without approaching a speaker. The behavior of the females was observed on a monitor using a wide-lens video system with infrared light source (Fuhrman Diversified, Inc). We used two-tailed exact binomial

probability tests to evaluate if the responses of females deviated from the 1:1 distribution expected if the experimental stimuli were ignored.

Males – evoked vocal response

In 2003, between July and August, calling males were captured at their breeding sites and brought to the lab where we tested them in evoked vocal response experiments using the same stimuli used in the phonotaxis experiments with females. Each male was placed inside individual acoustically-isolated chambers (30.5 cm x 46 cm x 30.5 cm) following Bosch et al. (2000; 2002). Males were placed with sufficient water to call in plastic bags previously shown to be acoustically transparent by Ryan & Rand (1998). We broadcast the experimental stimuli using a small, wide-frequency range speaker (Cambridge SoundWorks Inc., Ensemble IV) and recorded the response of the males with a Radio Shack miniature microphone into a Sony WM-D6 cassette recorder. The speaker was positioned in front of the male at about 15 cm, and the microphone was oriented to the calling frog but perpendicular to the speaker to maximize the difference in amplitude between the calls of the focal male and those played back. We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 µPa) at 0.5 m measured by a GenRad sound pressure level meter (model 1982). This sound pressure level is equivalent to the one used in the experiments with the females (82 dB at 1.25 m). We stimulated the males with a túngara frog chorus recorded on 2 October 1990 in the same population. Once a male was calling, the chorus was turned off and it was tested singly. After a male finished a test, it was required to call again before starting the

next test. Males were tested until they participated in all the experimental stimuli or stopped calling.

Two sets of experiments were conducted: (i) males were presented with túngara frog mating calls followed by sounds indicating increased predation risk, as in the female phonotaxis experiments above, and (ii) males were presented with the experimental stimuli without mating calls. In the first set of experiments, half of the males were exposed to synthetic whines for 1 min, silence for 1 min, and whines with one of the experimental stimuli appended to it for 1 min. The other males in this experiment were exposed to the whine followed by the experimental stimuli for 1 min, silence for 1min, and the whine by itself for another 1 min. We changed the order of presentation of the stimuli from one male to next one.

In the second set of experiments, we presented each experimental stimulus independently without being appended to a whine. Male túngara frogs are more permissive in their responses to signal variation than females (Bernal et al. 2007), and it is possible that a whine followed by any sound will elicit a response. To account for this, and potentially detect more subtle responses of males to sounds that represent increased hazard, we investigated their calling responses to the experimental stimuli alone. To do so we waited until a male was calling spontaneously and we then broadcast one of the experimental stimuli without a whine, and only once. We recorded the calling behavior of the males 15 sec before and after the presentation of the stimuli.

We digitized the tapes using CoolEdit 2000 (Syntrillum Software Corporation), at 44.1 kHz sampling rate and 16 bits/sample. Using this software we also measured the

latency time to the first call following stimulus broadcast (from the beginning of the presentation of the stimuli), number of whines and number of chucks during the presentation of the whine alone and the whine followed by the experimental sounds. We then performed a Principal Component Analysis (PCA) combining those three variables for each treatment, and used the score on the first component as a measurement for overall calling response. In this way, for each male we obtained a response score for calling in the presence of the call alone and calling in the presence of the call associated with the experimental stimuli.

In the experiments in which a single experimental stimulus was presented without a whine, we simply counted the number of calls because males rarely added chucks to their calls. Production of whines without chucks is common in males calling isolated from other calling males (Ryan 1985). We used Wilcoxon signed-rank tests to compare the calling response before and after the presentation of each experimental stimulus.

Comparison between females and males

To contrast the responses of the sexes to the experimental stimuli appended to mating calls, we converted the vocal response of males based on the load to the first components of the PCA into a response score that could be compared to the response scores of the females (e.g. choice for whine or for whine with the experimental sound appended). In each trial, the stimulus with the highest calling response was assigned as the one chosen. We compared the number of males and females that responded to each experimental pair of calls using two-tailed Fisher Exact tests.

4.3. Results

The results of all the experiments are summarized in Tables 4.1, 4.2 and 4.3. Female túngara frogs preferentially approached the mating call that was not associated with a predation attack or an approaching frog-eating bat (Table 4.1). Whines followed by the call of *L. pentadactylus* or the long chuck, however, were as attractive as a whine by itself.

Table 4.1. Túngara frog female phonotatic responses when presented with identical mating calls (whine) in which one is followed by a sound associated with increased predation risk (ES). The columns indicate the number of females that approached a whine alone or approached the whine followed by the experimental stimulus. The Binomial probability is shown in the last column and $p < 0.005$ are in bold.

Experimental Stimuli (ES)	Whine	Whine + ES	Binomial probability
Rustle	17	3	**0.001**
Splash	15	5	**0.015**
Bat wing-beats	18	2	**<0.001**
L. pentadactylus	13	7	0.074
Long chuck	8	13	0.097

Males were more responsive to calls associated with increased predation risk than to a single whine. Calls with the experimental stimuli appended to them elicited more calls, more chucks and shorter latency to response (Table 4.2). The first principal component from the PCA, used to reduce the variables to a single overall measurement of calling response, explained over 88% of the total variation in all treatments (88.31-89.46%, eigenvalue > 1.7). Overall calling response was significantly higher to whines

associated with the sound of bat wing-beats, rustle and the long chuck. Males, however, did not significantly increase their calling response when presented with the call of *L. pentadactylus* or the splash. In all the variables we measured, however, there were trends to increase calling response to both stimuli: males increased the number of calls and chucks, and also decreased the time to their first response when exposed to the sounds associated with increased danger.

Table 4.2. Calling response of male túngara frogs, *Physalaemus pustulosus*, to playbacks of a single mating call (whine) and a call followed by a sound representing increased predation risk (whine + ES). Mean (SEM) is presented. The Wilcoxon signed-rank tests shown is based on Principal Component Analyses for each treatment combining number of calls, number of chucks and latency time. For details about the procedure see the methods in the text. $P < 0.05$ are shown in bold.

Experimental Stimuli (ES)	Number of calls		Number of chucks		Latency time (s)		Wilcoxon signed-rank tests		
	whine	whine + ES	whine	whine + ES	whine	whine + ES	*Z*	*P*	*N*
Rustle	22.25 (1.90)	29.00 (0.98)	28.15 (4.05)	44.80 (4.93)	4.62 (2.04)	1.32 (0.06)	-2.70	**0.007**	10
Splash	24.22 (1.68)	25.77 (3.21)	35.44 (3.29)	45.11 (6.25)	2.91 (0.99)	1.31 (0.08)	-1.00	0.314	9
Bat wing-beats	23.80 (1.77)	32.30 (1.30)	32.20 (3.91)	60.10 (5.39)	2.08 (0.36)	1.96 (0.63)	-2.70	**0.007**	10
Leptodactylus pentadactylus	22.15 (1.59)	23.80 (2.72)	28.25 (3.70)	40.1 (5.82)	5.21 (2.05)	2.31 (1.03)	-1.78	0.074	10
Long chuck	21.85 (1.99)	32.30 (0.99)	28.00 (2.42)	64.70 (2.90)	6.31 (3.33)	1.74 (0.33)	-2.80	**0.005**	10

The effect of the experimental stimuli was different when these sounds were presented without the whine. Calling males exposed to sounds associated with increased danger, without the whine, showed a tendency to reduce the number of calls produced (Table 4.3). Males only significantly reduced their calling behavior in response to the sound of the bat wing-beats, however.

Table 3. Calling behavior of male túngara frogs, Physalaemus pustulosus, during 15-sec intervals before and after the presentation of sounds representing an increased risk of predation alone. N = 9 individuals in all treatments.

Experimental Stimuli (ES)	Number of calls		Wilcoxon signed-rank test	
	Before	After	*Z*	*P*
Rustle	3.33 (0.64)	1.89 (0.86)	-1.057	0.291
Splash	3.89 (0.65)	2.11 (0.89)	-1.442	0.149
Bat wing-beats	3.56 (0.55)	1.00 (0.60)	-2.264	**0.024**
L. pentadactylus	3.11 (0.66)	2.00 (1.12)	-1.016	0.310
Long chuck	3.78 (0.62)	2.22 (1.02)	-1.270	0.204

Females approaching a mate and calling males significantly differed in their responses to all stimuli with the exception of the whine followed by the call of *L. pentadactylus* (rustle: $P < 0.001$, splash: $P = 0.014$, bat wing-beats: $P < 0.001$, *L. pentadactylus*: $P = 0.122$, long chuck: $P = 0.032$). In all cases a higher proportion of

males than females responded to the mating calls associated with increased danger, and to the long chuck.

4.4. Discussion

Our results show differences between reproductively active female and male túngara frogs in their response to sounds associated with increased predation risk. While several studies have shown that some prey species respond to acoustic cues signaling the presence of a predator (Baxter et al. 2006; Hendrie et al. 1998; Jones et al. 2002; Phelps et al. 2007), to our knowledge this is the first study to reveal sexual differences in these responses.

Females avoided mating calls associated with sounds that indicate a predation attack but did not evade those coupled with the call of a common predator, *L. pentadactylus*. This behavior suggests that females perceived no threat when hearing calls of *L. pentadactylus*. Calling *L. pentadactylus* probably do represent a serious hazard even though they are also engaged in attracting mates. They are a voracious predator of túngara frogs (Ryan et al. 1981) and as in other frog-eating frogs males probably feed while calling (Schwartz et al. 2000). We are confident that females do not mistakenly recognize the call of *L. pentadactylus* for a túngara frog call; the two calls differ in the direction of their frequency sweeps and túngara frogs are sensitive to this call parameter (Rose et al. 1988). It is possible that female *P. pustulosus* from the population we sampled have had little experience with this predator. Where we collected females for this study, *Leptodactylus pentadactylus* are not as abundant as in other areas such as the

site studied by Ryan et al. (1981). In other species response to predators varies with predator density (e.g. guppies, Godin & Briggs 1996; damselflies, McPeek 1990; salamanders, Storfer & Sih 1998), and this relationship could be tested in túngara frogs.

Most of the sounds we used in our study affected female phonotaxis. This effect could be due to the experimental sounds acoustically interfering with the mating call. The fact that the long chuck did not alter the responses of females suggests that this may not be the case. If long, loud sounds following the whine interfere with the call, the long chuck would elicit the same effect as the other experimental stimuli we presented. It seems more likely that decrease in attractiveness is due to the association of the whine with our experimental sounds. In our tests using risk related sounds, female túngara frogs were presented with two identical calls, which should therefore offer equal benefits. This balance was likely disturbed when a potential cost was associated with one of the calls.

Studies examining the influence of risk on reproductive activity have generally concluded that predators reduce mating behavior of their prey (Dill et al. 1999; Johnson & Basolo 2003; Sih et al. 1990; Tuttle & Ryan 1982; but see Schwartz et al. 2000). Male *P. pustulosus* reduce their calling activity when exposed to visual cues imitating an approaching frog-eating bat (Tuttle et al. 1982). We expected similar responses to acoustic cues associated with high predation risk, in particular to the sound of an approaching bat. In our study, however, male túngara frogs behaved, contrary to our predictions, as they increased calling in response to those mating calls followed by sounds that represent increased danger of predation. Our results, however, suggest that male túngara frogs do not perceive the sounds we presented as indicators of increased

hazard as they did not cease calling. Whether or not these stimuli were associated with a whine, they did not result in decreased calling with one exception. That exception is the wing-beats of bats. By themselves they did result in decreased calling, but not when they followed a whine. Túngara frog males may be using the calls of other males to further assess the level of predation risk and modulate their response to sounds that otherwise would be perceived as a threat. In accord with this idea, male túngara frogs that cease calling following the release of a bat model or a disturbance, resume calling faster when hearing conspecific calls than in silence (Jennions & Blackwell 1992; Phelps et al. 2007).

It is clear from our results that reproductively active male and female *P. pustulosus* differ in their predator avoidance strategies when using acoustic cues as proxies for increased predation risk. Females behaved more cautiously than males suggesting that the sexes balance the risk of predation and the cost of cautious mating strategies differently. In our experiments, since females are presented with a two-choice paradigm there is no cost of losing mating opportunities for females when avoiding sounds related to increased hazard. For males, in contrast, reducing or ceasing their calling response has a direct negative impact in attracting females. This situation reflects the scenario for both sexes in nature. In túngara frogs the operational sex ratio is strongly male biased and thus females have multiple opportunities to mate while males have fewer chances of attracting a mate (Ryan 1985). Females actively choose a mate from a pool of calling males while males vocally compete with neighbor males to attract a mate. Intense intrasexual competition to obtain a mate combined with high predation risk probably led males to tune their response to predators in a finer way than females.

Females might also be more sensitive to the sounds indicating potential threats than males because of the vulnerability of moving towards a potential mate, and the possibility of adjusting their behavior without incurring in high costs. A female, for instance, can alter her path and choose another male that is not as close to the perceived threat. Calling males, however, must call to attract a mate. Alternative mating strategies have not been described in túngara frogs despite the extensive work done on this species. This lack of alternative behaviors for males may also underlie their willingness to engage in risky behaviors.

Few studies have investigated the use of acoustic signals in anurans to detect predators or other dangers in spite of the relevance of this sensory modality in this group (Schwartz et al. 2000; Grafe et al. 2002). This study is the first demonstration that *P. pustulosus* females use acoustic cues associated with predators and predation attacks to modulate their mating behavior, and the first study to demonstrate different responses in males versus females in response to acoustic cues signaling predation risk.

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Chapter 5: Sexual Dimorphism in Behaviour? Task Differences Underlie Sex Differences

Abstract. Differences in behaviour, morphology and physiology between species are often dwarfed by differences between the sexes. Such differences have been well demonstrated in sexual communication. In many animals, males and females invest differentially in reproduction, although there are exceptions. This difference often results in reproductive strategies in which selection favors males who increase their number of mates while females are under selection to increase mate quality. Consequently, females usually are more selective in their response to variation in signals than males. But most inferences about sex differences in signal selectivity are derived from tests of males and females performing different tasks. Here we report that presumed sexual differences in túngara frogs (*Physalaemus pustulosus*) in response to mating signals result from task differences and not sex differences. Male are less selective in their vocal responses to advertisement calls than females in responding with phonotaxis to the same calls; the canonical mating behaviour for each sex. When males exhibit phonotaxis to the same calls, however, their selectivity mimics that of females during phonotaxis, and is significantly more selective than male vocal responses to the same calls. Analogous task differences might confound sex differences in other systems, thus we suggest consideration of the behavioural plasticity of gender as well as its stereotypy.

Differences between females and males are inherently interesting, and differences in reproductive behaviour, in particular, have attracted special consideration and debate (Darwin 1871; Gray 1992; Roughgarden et al. 2006). In sexual communication such differences arise from an asymmetry between the sexes in fitness costs of recognition errors of mating signals; costs are usually higher for females than males. There are two types of recognition errors: (i) misidentification, when an inappropriate signal is falsely accepted as appropriate, and (ii) missed opportunity, when an appropriate signal is falsely rejected as inappropriate. Females are under strong selection to avoid misidentification (e.g., mating with the wrong species) while there is less cost for missed opportunity (being able to find a mate). Alternatively, males are under strong selection to avoid missed opportunities (passing up a mate) while there is usually less cost for misidentification (mating with the wrong species). Males and females, however, characteristically perform different behaviours in response to sexual signals complicating direct comparisons between the sexes. For example, in acoustic advertisement males often compete with each other vocally while females use the call or song to locate and evaluate mates. This conundrum of task and sex occurs in many systems, obscuring an important question: is there an intrinsic sexual difference in how males and females respond to conspecific signals, or is there a difference in the expression of sex-related behaviors to those signals in males and females?

Here we investigate a Neotropical frog in which males produce calls intended to attract females and, at the same time, deter rivals. Male túngara frogs, *Physalaemus pustulosus*, aggregate to advertise at breeding ponds drawing females and males to the

chorus. At these aggregations females have higher odds of mating than males; there are usually at least three times as many males as females (Ryan 1985). Females use the male mating call to locate and assess mates. Her approach to the call, phonotaxis, indicates her decision. Male túngara frogs typically respond to calls of other males vocally, by calling back to them. There are differences between the sexes in their selectivity to signal variation. Consistent with sexual selection theory, only a narrow range of signal variation elicits female phonotaxis while a much larger range of variation will elicit calling from males (Bernal et al. 2007). Although such differences are often described as 'sex differences' (e.g. Searcy & Brenowits 1988; Ratcliffe & Otter 1996; Dabelsteen & Pederson 1988), the differences could be task-specific independent of sex.

Male túngara frogs (Ryan 1985), like males of many other species (Bee 2007; Kaspi &Yuval 1999; Mountjoy & Lemon 1991), use signals of other males to locate breeding sites. Here we quantify the evoked vocal response of males, and the phonotaxis behaviour of both males and females to signal variation to test the alternative hypotheses that (i) differences in response are due to sex and are independent of task versus (ii) differences in response are due to task and are independent of sex.

The mating calls of túngara frogs consist of a whine, a low-frequency sweep, which may be followed by zero to seven secondary components (chucks). The whine is necessary and sufficient to elicit a behavioural response in females and males (Ryan 1985). We examined call recognition in response to variants of the whine (without chucks). We first explored call recognition altering specific parameters in the frequency domain of the signal. Frequency composition of mating calls in frogs is essential for call

recognition in many species, including túngara frogs (Gerhardt & Huber 2002; Wilczynski et al. 1995). In the context of phonotaxis, females and males exposed to white noise controls, that mimic only the call's temporal pattern, do not respond to the stimuli. Calling males, in contrast, vocally respond to the whine-like noise (Fig. 5.1a). Calling males not only recognize such stimulus, they increase their vocal response significantly more to the noise than to the conspecific call (Wilcoxon signed-ranked test Z = -2.449, P = 0.014).

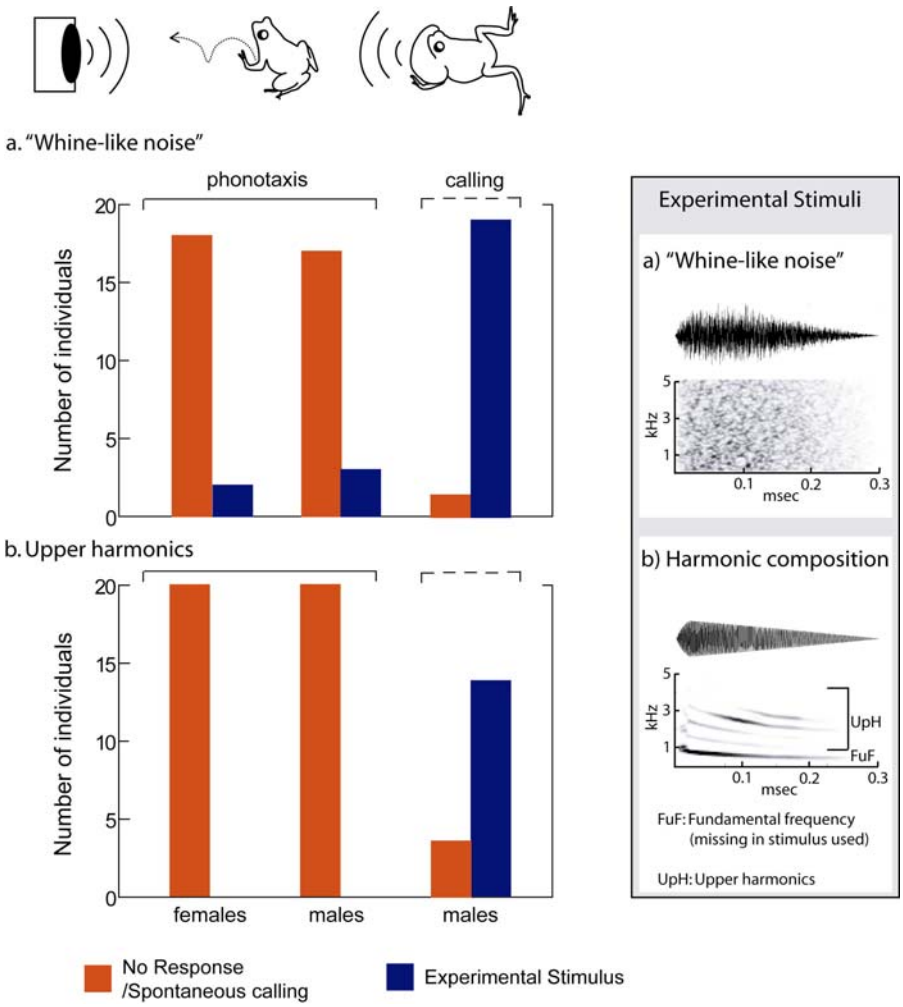

Figure 5.1. Intrinsic similarities between the sexes. Number of male and female túngara frogs responding to calls with altered frequency characteristics. a, Responses to "whine-like noise". b, Responses to whines with only upper harmonics. In both sets of experiments females and males performing phonotaxis agree in their responses (Fisher Exact test, P = 1 all comparisons), but not with the ones of calling males (Fisher Exact test, P < 0.0001 all comparisons). Red bars: Lack of response in phonotaxis or higher vocal response during spontaneous calling. Blue bars: positive phonotaxis or higher vocal response to the experimental stimulus. Graphical representations of the stimuli used are presented in the box on the right.

A parallel pattern emerges in recognition of calls with altered harmonic composition. The majority of the energy of the whine is distributed in five harmonic frequencies in which the fundamental frequency contains about 50% of the energy. We evaluated how túngara frogs perceive whines without the fundamental frequency. When performing phonotaxis, males and females are concordant in their lack of response to the upper harmonics of the whine, and are discordant with the responses of calling males (Fig 5.1b). Thus, as predicted, females exhibiting phonotaxis are more discriminatory than calling males. This difference, however, disappears when we contrast the sexes performing the same task. These data reject the hypothesis that differences in signal selectivity between calling males and searching females are due to sex (independent of task) and support the hypothesis that the differences are due to task (independent of sex).

We further replicated these tests by determining if such task differences emerge in response to more subtle signal variation encountered in the wild. We conducted experiments contrasting the responses of the sexes to natural calls. We presented the frogs with a synthetic call, the average whine of the population, and the calls of four randomly chosen males. Two calls elicited similar responses in both sexes and task; the natural call elicited more calling and preferential phonotaxis compared to the synthetic call (Fig. 5.2a,b). In response to the other two calls, however, there were task but not sex differences (Fig. 5.2c,d). Males called more to the natural calls compared to the synthetic call, while both males and females preferred the synthetic call (call male C) or showed no phonotactic preferences (call male D).

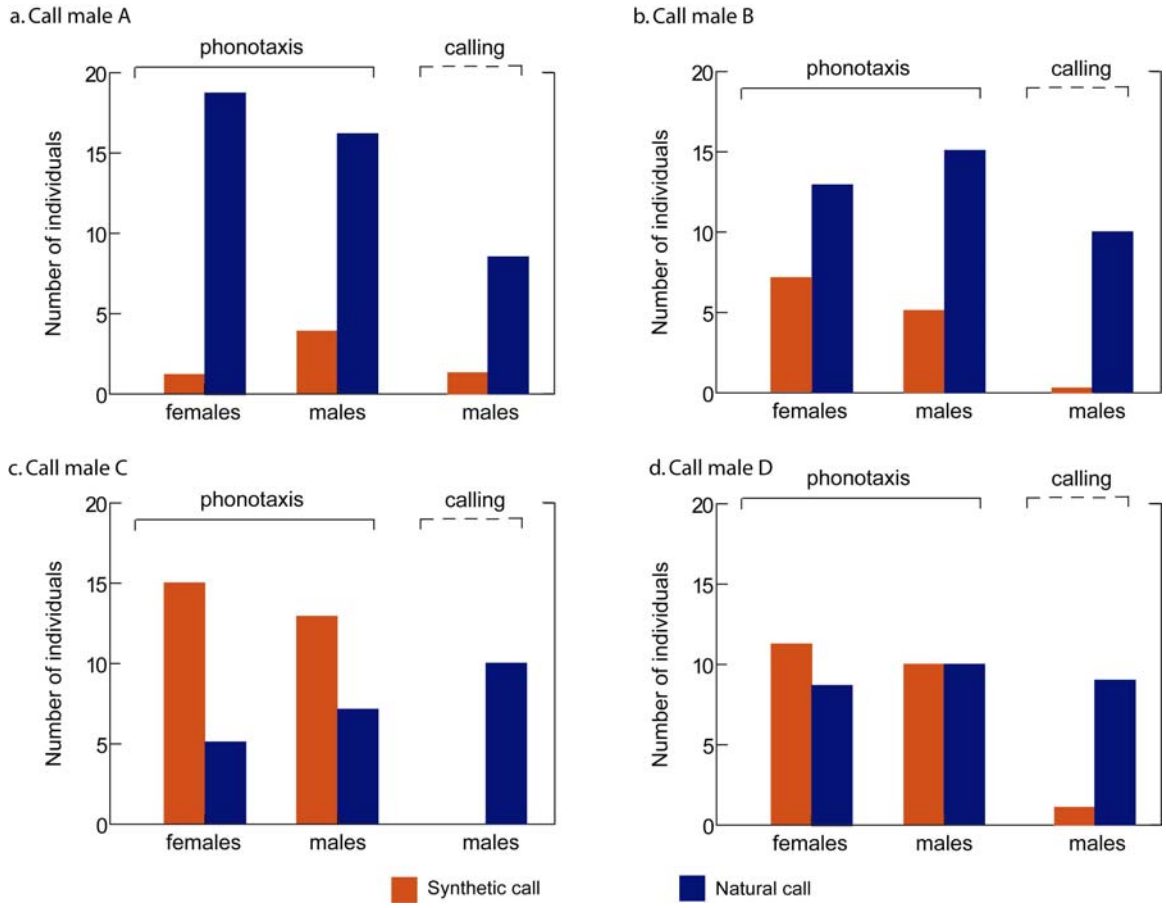

Figure 5.2. Task differences underlying sex differences extent to natural variation. Number of male and female responding to synthetic calls (red bars) and natural calls (blue bars). The responses of both sexes to the calls of male A and B, independent of task performed, are not statistically different (Fisher Exact test, $P > 0.203$ all comparisons). In response to the calls of male C and D there are no between-sex differences in phonotactic responses (Fisher Exact test, $P > 0.731$ all comparisons). But the phonotactic responses of both sexes are different from the ones of calling males (Fisher Exact test, call C: phonotaxis females vs. calling males $P < 0.001$, phonotaxis males vs. calling males $P = 0.001$; call D: phonotaxis females vs. calling males $P = 0.017$, phonotaxis males vs. calling males $P = 0.04$).

We performed a total of thirteen sets of experiments in which both sexes performed phonotaxis and males called back in response to the stimuli. Females and males exhibiting phonotaxis behaved similarly to one another and more selectively than calling males in the six cases in which there were differences in response. While calling males respond to a broad range of stimuli, frogs of either sex approaching a call respond only to a subset of those. Calling males never behaved more selectively than frogs in phonotaxis.

There is no question that across many taxa, including our own, the sexes are different. These differences extend to the realm of behaviour, and sex differences in reproductive behaviour have been a cornerstone of behavioural evolution. But the reproductive tasks of males and females often differ, and sex differences have often been interpreted from males and females performing different behaviours. Our study shows that apparent differences between the sexes emerge from differences in the tasks themselves. Thus the differences between the sexes in their stimulus selectivity might be due to task-specific responses, which do not indicate inherent sexual differences in behavioural, neural or cognitive aspects of stimulus processing but instead suggest behavioural plasticity.

The sexes are often conceived as a product of different templates shaped by distinct developmental hormonal processes. Males and females, however, might show differences that are the product of typically expressing different behavioural strategies rather inherent differences in their templates. In this view, theoretical arguments regarding selection for reproductive strategies still hold, but are applicable to the

behaviors expressed rather than to the sex of the individual expressing them. In some species flexibility in such expression might overshadow the differences between the sexes (Crews & Fitzgerald 1980; Aubin-Horth et al 2007). In examining the nature of the sexes, behavioural similarities and differences between males and females are central to both the mechanisms understanding common neural pathways and the evolution of behavioural strategies. Future studies should consider the behavioural plasticity of gender as well as its stereotypy (e.g. Crews 1988).

5.1. Methods

Collection and experimental stimuli. Túngara frogs were collected in Gamboa, Panama, at breeding sites near the research facilities of the Smithsonian Tropical Research Institute. Females were collected in amplexus while males were found either paired with females or calling at the ponds. We brought the frogs to the lab and tested them singly. The synthetic stimuli were generated by shaping sine waves using the software developed by J. Schwartz (Pace University at Pleasantville, NY; sample rate 20 kHz and 16 bit) using the mean values of the parameters of the calls in the population (based on the recordings of 50 males). Call parameters were calculated using batch processing programs in the software package Signal (Engineering Design, Belmont, MA). Information on the recordings, call parameters used and the synthesis procedure can be found in Ryan & Rand (2003). To generate the “whine-like noise” we synthesized white noise and filtered it to match the mean amplitude envelope of the call. Average versions of each harmonic frequency of the whine were synthesized using the mean

contour of each harmonic sweep. Calls including only the upper harmonics were produced adding the frequency contour of harmonics two to five maintaining their original relative amplitude. The natural calls used in this study were recorded in the same population where the test frogs were collected following standard procedures (Heyer 1994).

Phonotaxis. Females and males (20 individuals per sex on each experiment, total of 240 choice tests) were tested in a 1.8 m x 2.7 m sound attenuation chamber (Acoustic Systems, Austin, TX). We broadcast the experimental stimuli antiphonally from speakers in the center of walls opposite one another such that the peak amplitude of the call at the center of the arena, where the frog was released, was 82 dB SPL (re. 20 μPa). A response was scored when the frog approached a speaker within 10 cm, excluding approaches along the walls of the arena. We scored a "no response" if the frog either remained motionless for 5 min after being released, stopped moving for 2 min at any time during the experiment, or did not approach any speaker in 15 min. Tests to discern absence of response due to lack of motivation rather than lack of attraction to the call were performed. A complete description of the testing chamber and details of the protocol can be found in Ryan & Rand (1999).

Evoked vocal response. Calling males (whine-like noise: 20 males, upper harmonics: 18 males, natural calls: 10 males per experiment; total of 78 evoked vocal response tests) were tested in individual acoustically-isolated chambers (30.5 cm x 46 cm x 30.5 cm). Each male was placed in a plastic bag (previously shown to be acoustically transparent) inside the acoustic chamber that contained a Radio Shack miniature microphone and a

small, wide-frequency range speaker (Cambridge SoundWorks Inc., Ensemble IV). We stimulated the males to call with a recording of a high density túngara chorus, and once a male began to call we initiated the playback experiments. Each test consist of a set of five 60-second intervals: i) Control stimulus: synthetic average whine, ii) Silence, iii) Experimental stimulus: whine-like noise, call with upper harmonics only, or natural call, iv) Silence, and v) Control stimulus: synthetic average whine (Bosch et al 2000, Bernal et al 2007). Only cases in which males called during both control stimuli were included in the analysis to eliminate cases of no response due to lack of motivation. We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 μPa) at 0.5 m measured by a GenRad sound pressure level meter model 1982. The overall response of males was characterized using the first component of a principal component analysis (PCA) combining the total number of whines and total number of chucks for each interval during the test (in all experiments PC1 explained >90.1% of the variation). We converted the vocal response of males into a binary response equivalent to the one of females (i.e. response, no response). If a male called more during the presentation of the stimulus than during the silent intervals before and after it, his behaviour was scored as a response. A “no response” was scored when the baseline calling (during silence) was equal or higher than the response to the experimental stimuli. In the case of the experiments with the natural calls, a preference for a stimulus was assigned to the call with the highest calling response.

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